Study of Antimicrobial, Antioxidant and Thrombolytic activity of the fruit skin of *Spondias pinnata*

A Dissertation Submitted to

The Department of Pharmacy, East West University

In Partial Fulfillment of the Requirement

For the Degree of Bachelor of Pharmacy

Supervised by

Kh. Tanvir Ahmed

Lecturer, Department of Pharmacy

East West University

Submitted by

Mohammad Kawsar Manik

ID No# 2008-3-70-034

Date: 19th June, 2012

Endorsement by Head of the Department

This is to certify that the dissertation entitled "Study of Thrombolytic, Antioxidant and Antimicrobial Activity of *Spondias pinnata* (Skin)." is a bona-fide research work done by Mohammad Kawsar Manik under the guidance of Kh. Tanvir Ahmed, Lecturer, Department of Pharmacy, East West University .

Dr. Sufia Islam
Associate professor and chairperson
Department of Pharmacy
East West University

Certificate by the Supervisor

This is to certify that the dissertation entitled "Study of Thrombolytic, Antioxidant and Antimicrobial Activity of *Spondias pinnata* (Skin)." is a bona-fide research work done by Mohammad Kawsar Manik, in partial fulfillment of the requirement for the Degree of Bachelor of Pharmacy. It is a record of original research work carried out by me during fall 2011 to spring 2012 under the supervision and guidance of me.

Kh. Tanvir Ahmed,

Lecturer,

Department of Pharmacy

East West University

Declaration by the Candidate

I, Mohammad Kawsar Manik, hereby declare that this dissertation entitled "Study of Thrombolytic, Antioxidant and Antimicrobial Activity of *Spondias pinnata* (Skin)." submitted to the Department of pharmacy, East West University, in the partial fulfillment of the requirement for award of the degree of bachelor of pharmacy. It is a record of original research work carried out by me during fall 2011 to spring 2012 under the supervision and guidance of Kh. Tanvir Ahmed, Lecturer, Department of Pharmacy, East West University and it has not formed the basis for the award of any other degree/diploma fellowship or other similar title to any candidate of any university.

Mohammad Kawsar Manik
ID No#2008-3-70-034
Department of Pharmacy
East West University

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Dedicated

TO

My Parents

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Abstract

The skin of *Spondias pinnata* fruit (family - Anacardiaceae) was subjected to clinical and biological examination in the study. The powdered whole plant was extracted with ethanol at room temperature (cold extraction) and the extract was partitioned between n-hexane, dichloromethane,and Ethyl acetate fractions.

The crude ethanoic extract and fractions were preliminary screened for their various phytochemical and pharmacological properties.

The antimicrobial activities of extracts were examined. The crude extract produced strong activity against most of the organisms that's why fraction of the crude extract was subjected to the antimicrobial test. The zones of inhibition produced by the n-hexane, dichloromethane, ethyl acetate and aqueous soluble partitionates of the ehanolic extract were ranged from 0-15 mm, 7-14 mm, 0-17 mm and 0-12 mm respectively at a concentration of 400µg/disc.

Antioxidant property of the various fraction of the plant was determined by using five methods and good antioxidant activity was found for all the fractions.

The study examined the thrombolytic potential of different extracts of *S. pinnata* skin *in vitro.* It is found that among all the samples only n-hexane fraction of the ethanolic extract of the plant has thrombolytic activity compared to the standard. Again, only single concentrations of the extracts were examined. So, finding of the dose related trend, if any, of thrombolytic activity can be subjected to further study.

Key Words: Spondias pinnata, Antimicrobial, MIC, MBC, Antioxidant.

Chapter-1: Introduction

1.1. Rationale and Objective of the Work

Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago (Solecki & Shanidar, 1975). From that point the development of traditional medical systems incorporating plants as a means of therapy can be traced back only as far as recorded documents of their likeness. However, the value of these systems is much more than a significant anthropologic or archeologic fact. Their value is as a methodology of medicinal agents, which, according to The World Health Organization (WHO), almost 65% of the world's population has incorporated into their primary modality of health care (Farnsworth *et al.,* 1985).

The goals of using plants as sources of therapeutic agents are (Daniel *et al.,* 2003)

a) To isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine.
b) To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, verapamil, and miodarone, which are based, respectively, on galegine, Δ9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, khellin, and khellin;
c) To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, yohimbine; and
d) To use the whole plant or part of it as an herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, etc.

The number of higher plant species (angiosperms and gymnosperms) on this planet is estimated at 250,000 (Ayensu & DeFilipps, 1978), with a lower level at 215,000 (Cronquist, 1981; Cronquist, 1988) and an upper level as high as 500,000 (Tippo & Stern, 1977; Schultes, 1972). Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (Verpoorte, 2000).

With high throughput screening methods becoming more advanced and available, these numbers will change, but the primary discriminator in evaluating one plant species versus another is the matter of approach to finding leads. There are some broad starting points to selecting and

obtaining plant material of potential therapeutic interest. However, the goals of such an endeavor are straightforward.

Chemical diversity of secondary plant metabolites that results from plant evolution may be equal or superior to that found in synthetic combinatorial chemical libraries. Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. *Dutura has long been associated with the worship of Shiva, the Indian god*). Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic. Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants. Plant resources (E.g. *Angiosperm*, *Gymnosperm*, *Seedless vascular plants, Bryophytes*) for new medicine. The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases. To identify alternative and complementary medicine. To reduce the toxicity of drug therapy especially toxicity reduction of synthetic and semi synthetic drugs. To find the lead compound diversification to treat various diseases.

It was estimated that in 1991 in the United States, for every 10,000 pure compounds (most likely those based on synthesis) that are biologically evaluated (primarily *in vitro*), 20 would be tested in animal models, and 10 of these would be clinically evaluated, and only one would reach U.S. Food and Drug Administration approval for marketing. The time required for this process was estimated as 10 years at a cost of $231 million (U.S.) (Vagelos, 1991). Most large pharmaceutical manufacturers and some small biotechnology firms have the ability to screen 1,000 or more substances per week using high throughput *in vitro* assays. In addition to synthetic compounds from their own programs, some of these companies screen plant, microbial, and

marine organisms. The work described in this dissertation is an attempt to identify the biological activity of indigenous medicinal plants, viz., *Spondias pinnata* (Fam. Anacardiaceae) and to evaluate the possible toxicological and microbiological profiles of the crude extracts.

Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. *Dutura has long been associated with the worship of Shiva, the Indian god*). Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic. Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants. Plant resources (E.g. *Angiosperm*, *Gymnosperm*, *Seedless vascular plants, Bryophytes*) for new medicine. The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases. To identify alternative and complementary medicine. To reduce the toxicity of drug therapy especially toxicity reduction of synthetic and semi synthetic drugs. To find the lead compound diversification to treat various diseases.

1.2 History of traditional herbal medicine in Bangladesh

Traditional Medicine is the medicine or treatment based on traditional uses of plants, animals or their products, other natural substances (including some inorganic chemicals), religious verses, cultural practices, and physical manipulations including torture. As this system of medicine has been in use almost unchanged generation after generation throughout the ages for the treatment of various physical and psychological diseases, it is called traditional. Most of the times, the type, preparation, and uses of traditional medicines are largely influenced by folklore customs and the cultural habits, social practices, religious beliefs and, in many cases, superstitions of the people who prescribe or use them.

The earliest mention of traditional medicine is found in *Rigveda,* the oldest repository of knowledge in this subcontinent. Later *Ayurveda,* developed from the *Vedic* concept of life, became the important source of all systems of medical sciences. In course of time it became a part of culture and heritage of the people of the Indian subcontinent.

Traditional medicine involves the use of both material and non-material components. The material components invariably comprise parts or organs of plants and their products. They also consist of animal organs, minerals and other natural substances. The non-material components, which constitute important items of religious and spiritual medicines, include torture, charms, magic, incantations, religious verses, amulets and rituals like sacrifices, appeasement of evil spirits, etc.

Treatments in traditional medicine are carried out by internal and external application of medicaments, physical manipulation of various parts of the body, performing rituals, psychological treatment, and also by minor surgery.

Ayurvedic medicinal preparations consist mainly of plant materials in the form of powders, semi-solid preparations, decoctions, elixirs and distillates. Many of them also contain inorganic chemical substances, minerals and animal products. Alcoholic extracts and alcoholic solutions of the ingredients, tinctures and elixirs are also frequently used in *Ayurvedi*c medicine. Whole plants or their powders or pastes or products and their extracts, infusions, decoctions and distillates constitute the major constituents of *Unani* medicine. Minerals, inorganic chemicals and animal products are also frequently used in preparing these medicines.

For hundreds of years, the medical knowledge in Bangladesh is termed as Ayurveda. Ayurveda remains an important system of medicine and drug therapy in Bangladesh. Plant alkaloids are the primary active ingredients of Ayurvedic drugs. There are also some other ingredients which are responsible for producing therapeutic effects.

Today the pharmacologically active ingredients of many Ayurvedic medicines are being identified and their usefulness in drug therapy being determined and many of those are used in the modern medical therapy.

As only a certain percentage of plants are used in traditional medicines, it is roughly estimated that of the discovered 17,000 species, nearly 3,000 species are used in medicinal field. Some crude drugs used as medicine in Bangladesh are reported in following table (Table 1.1).

Table 1.1: Some crude drugs used as medicine in Bangladesh

Common name	Botanical name	Uses
Amla	*Emblica officinalis*	Vitamin - C, Cough, Diabetes, cold, Laxative, hyper acidity.
Ashok	*Saraca asoca*	Menstrual Pain, uterine, disorder, Deiabetes.
Bael / Bilva	*Aegle marmelous*	Diarrrhoea, Dysentry, Constipation.
Chiraita	*Swertia chiraita*	Skin Desease, Burning, censation, fever.
Kalmegh/ Bhui neem	*Andrographis paniculata*	Fever, weekness, release of gas.
Long peeper / Pippali	*Peeper longum*	Appetizer, enlarged spleen, Bronchities, Cold, antidote.
Pashan Bheda / Pathar Chur	*Coleus barbatus*	Kidny stone, Calculus.
Sandal Wood	*Santalum album*	Skin disorder, Burning, sensation, Jaundice, Cough.
Satavari	*Asparagus racemosus*	Enhance lactation, general weekness, fatigue, and cough.
Senna	*Casia augustifolia*	General debility tonic, aphrodisiac.
Tulsi	*Ocimum sanclum*	Cough, Cold, bronchitis,expectorand
Pippermint	*Mentha pipertia*	Digestive, Pain killer
Henna/Mehd	*Lawsennia iermis*	Burning, Steam, Anti Imflamatary
Gritkumari	*Aloe verra*	Laxative, Wound healing, Skin burns & care,Ulcer
Sada Bahar	*Vincea rosea*	Leaukamia, Hypotensiv, Antispasmodic , Atidot
Vringraj	*Eclipta alba*	Anti-inflamatory, Digestive, hairtonic
Neem	*Azardirchata indica*	Sdedative, analgesic, epilepsy, hypertensive
Anantamool/sariva	*Hemibi smus indicus*	Appetiser, Carminative, aphrodisiac, Astringent
Kantakari	*Solanum xanthocarpum*	Diuretic, Antiinflamatory, Appetiser, Stomachic
Shankhamul	*Geodorum denciflorum*	Antidiabetic

1.3.The plant family: Anacardiaceae

Anacardiaceae, the cashew family, includes approximately 800 species in 82 genera. Members of the family are cultivated throughout the world for their edible fruits and seeds, medicinal compounds, valuable timber, and landscape appeal. Some of the products of Anacardiaceae, including mango (*Mangifera indica*), pistachio (*Pistacia vera*), cashew (*Anacardium occidentale*), and pink peppercorn (*Schinus terebinthifolia*), are enjoyed worldwide while other notables such as the pantropical Spondias fruits, the marula of Africa (*Sclerocarya birrea*), and the Neotropical fruits of Antrocaryon are restricted to localized cultivation and consumption and are not generally transported far distances to larger markets.

1.3.1. Taxonomy

The exact definition of taxonomy varies slightly from source to source, but the core of the discipline remains: the identification, naming, and classifying of organisms. As points of reference, three recent textbook definitions are presented below:

1. Theory and practice of grouping individuals into species, arranging species into larger groups, and giving those groups names, thus producing a classification (Judd *et al.*, 2007)
2. A field of science (and major component of systematics) that encompasses description, identification, nomenclature, and classification. (Simpson, 2010)
3. The science of classification, in biology the arrangement of organisms into a classification. (Kirk *et al.*, 2008)

In 1759, Bernard de Jussieu arranged the plants in the royal garden of the Trianon at Versailles, according to his own scheme. That classification included a description of an order called Terebintaceae which contained a suborder that included Cassuvium (Anacardium), Anacardium (Semecarpus), Mangifera, Connarus, Rhus and Rourea. In 1789, Antoine Laurent de Jussieu, nephew of Bernard de Jussieu, published that classification scheme. Robert Brown described a subset of Terebintaceae called Cassuvlae or Anacardeae in 1818, using the herbarium that was collected by Christen Smith during a fated expedition headed by James Kingston Tuckey to explore the River Congo. The name and genera were based on the order with the same name that had been described by Bernard de Jussieu in 1759. The herbarium from that expedition contained only one genus from the family, Rhus. Augustin Pyramus de Candolle in 1824, used Robert

Browns name Cassuvlæ or Anacardeae, wrote another description of the group and filled it with the genera Anacardium, Semecarpus, Holigarna, Mangifera, Buchanania, Pistacia, Astronium, Comocladia and Picramnia. John Lindley described the "Essential character" of Anacardiaceæ, the "Cashew Tribe" in 1831, adopting the order that was described by Jussieu but abandoning the name Terebintaceæ. He includes the genera which were found in de Candolle's Anacardieæ and Sumachineæ: Anacardium, Holigarna, Mangifera, Rhus and Mauria.The genus Pistacia has sometimes been separated into its own family, Pistaciaceae, based on the reduced flower structure, differences in pollen, and the feathery style of the flowers. However, the nature of the ovary does suggest it belongs in the Anacardiaceae, a position which is supported by morphological and molecular studies, and recent classifications have included Pistacia in the Anacardiaceae.

1.3.2 Characteristics

The Anacardiaceae includes primarily trees and shrubs (rarely lianas or subshrubs), with resin canals and clear to milky exudate. The leaves are estipulate, usually alternate (rarely opposite or whorled), and may be simple or pinnately compound (very rarely bi-pinnate; *Spondias bipinnata*). The flowers are generally not highly conspicuous but are distinctive in having an intrastaminal nectariferous disc and apotropous ovules (an ovule with a raphe that is ventral when ascending and dorsal when descending). Morphological fruit diversity is exceedingly high with a myriad of types found in the family. Although the majority of the family has drupaceous fruits, many of these are variously modified for different mechanisms of dispersal and several other fruit types are also represented. Two genera, *Anacardium* and *Semecarpus*, have an enlarged edible hypocarp subtending the drupe. One species of *Anacardium*, *A. microsepalum*, lacks the hypocarp and grows in the flooded forests of the Amazon where it may be fish dispersed (Mitchell and Mori, 1987; J. D. Mitchell, pers. com.).

Water dispersal has been reported or purported for three genera, *Mangifera*, *Poupartiopsis*, and *Spondias*. The variety of mechanisms for wind dispersal include subtending enlarged sepals (*Astronium*, *Loxostylis*, *Myracrodruon*, *Parishia*), subtending enlarged petals (*Gluta*, *Swintonia*), trichome-covered margins on a globose fruit (*Actinocheita*), trichome-covered margins on a flattened fruit (*Blepharocarya*, *Ochoterenaea*), elm-like samaras with an encircling marginal wing (*Campylopetalum*, *Cardenasiodendron*, *Dobinea*, *Laurophyllus*, *Pseudosmodingium*,

Smodingium), samaras with a single wing (*Faguetia*, *Loxopterygium*, *Schinopsis*), dry samara-like syncarps (multiple fruit, *Amphipterygium*, *Orthopterygium*), dry achene-like fruit (*Apterokarpos*), and elongated ciliate pedicles of sterile flowers on a tumbleweed-like infructescence (*Cotinus*).

1.3.3 Distribution

Anacardiaceae are found worldwide in dry to moist, mostly lowland habitats, primarily in the tropics and subtropics but extending into the temperate zone. The family is native to the western hemisphere (from southern Canada south to Patagonia), Africa, southern Europe, temperate and tropical Asia, tropical and subtropical Australia, and most of the Pacific Islands. Anacardiaceae are absent from northern Europe, temperate and arid Australia, New Zealand, the Galapagos Islands, and extreme desert and high elevation habitats (although they can reach elevations as high as 3,500 m).

1.3.4 Anacardiaceae Species Available in Bangladesh

Anacardiaceae species found in all over the Bangladesh. Some of the plant of this sepsis is economically important for producing fruit and wood. Many of these plants are used as the remedy of many diseases e.g *Lannea grandis* is used in the treatment of urinary problem, *Mangifera indica* is used in the trestment of the influenza and *Spondias mombin is used as diuretic*. Anacardiaceae species found in Bangladesh which are medicinally important are shown in the table below-

Table 1.2: Anacardiaceae Species Available in Bangladesh

Scientific name	Genus	Local name	Medicinal uses
1. *Lannea grandis*	*Lannea*	Jikkha	Urinary problems. Leaf is taken as diuretic
2. *Mangifera indica*	*Mangifera*	Aame/ Theghace	Influenza, helminthiasis. The green fruit is used against influenza. The bark is used against helminthic infections

3. Lannea coromandelica	*Lannea*	Jiga/Jigar	Seminal problems. The bark is aadministered in cases of seminal weakness and excessive seminal emissions.
4. Spondias pinnata	*Spondias*	Amra	Used as fruit
5. Spondias mombin	*Spondias*	Amra	The fruit-juice is used as a febrifuge and diuretic.
6. Mangifera sylvatica	*Mangifera*	Himalayan Mango, Pickling Mango, or Nepal Mango	Influenza, helminthiasis.
7. Anacardium giganteum	*Anacardium*	Kazu badam	Anti-mycoses, antioxidant
8. Anacardium occidentale	*Anacardium*	Kazu badam	Used as fruit
9. Buchanania arborescens	*Buchanania*	Blume	Unknown
10. Schinus polygama	*Schinus*	Morich gach	Anti-inflammatory
11. Buchanania lanzan	*Buchanania*	Chirauli	Unknown
12. Choerospondias axillaris	*Choerospondias*	Lapsi	Unknown
13. Toxicodendron acuminatum	*Toxicodendron*	Bichuti	Anti-inflammatory, Analgesic
14. Spondias dulcis	*Spondias*	Amra	The fruit-juice is used as a febrifuge and diuretic.

(Md. Shahidullah, 2009)

1.4. Introduction to *Spondias pinnata*:

1.4.1. Taxonomic hierarchy of the investigated plant

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Super division	:	Spermatophyta
Subclass	:	Rosids
Order	:	Sapindales
Family	:	Anacardiaceae
Genus	:	*Spondias*
Species	:	*Spondias pinnata.*

1.4.2. Plant Description

Genus: *Spondias*

Species: *pinnata*

Family: Anacardiaceae

Local name	Country
Amra	Bangladesh
Caja-manga	Brazil
June plum	Bermuda and Jamaica
Quả cóc	Vietnam

Category: Ornamental tree

Height: 12-to18 m with upright growth.

Stem: Soft Wooded

Roots: Deep roots, Tap roots

Figure1.1: Picture showing different parts of *Spondias pinnata*

This highly ornamental tree is a rapid-grower reaching a height of 12-to18 m with upright growth. Deciduous in habit, its leaves turn bright yellow before falling in the cool season. The small white flowers are borne in large bunches with male, female and perfect flowers within each cluster. The long-stalked fruits dangle in bunches of a dozen or more.While green the fruit will fall to the ground over a period of several weeks. As they mature the skin turns golden-yellow with an orange-yellow pulp surrounding a single large spiny seed. When ripe they have a pleasant pineapple-like fragrance.

1.4.3 Growing conditions

Like the Mango, the tree thrives in humid tropical and subtropical areas growing up to 2 metres in a single growing season. It grows on all types of soil, as long as they are well drained. It has been noted that some trees can suffer from some nutritional disorders if the soil is too alkaline. Trees are cold sensitive when small and should be protected from serious frost and strong wind. Trees do best in full sun, but will produce some fruit in light shade. As a large and vigorous tree

they prefer not be planted underneath other large trees and unlike some mango varieties they are not too fussed on salt spray.

1.4.4 Uses as Food

The ambarella has suffered by comparison with the mango and by repetition in literature of its inferior quality. However, taken at the proper stage, while still firm, it is relished by many out-of-hands, and it yields a delicious juice for cold beverages. If the crisp sliced flesh is stewed with a little water and sugar and then strained through a wire sieve, it makes a most acceptable product, much like traditional applesauce but with a richer flavor. With the addition of cinnamon or any other spices desired, this sauce can be slowly cooked down to a thick consistency to make a preserve very similar to apple butter. Unripe fruits can be made into jelly, pickles or relishes, or used for flavoring sauces, soups and stews.Young ambarella leaves are appealingly acid and consumed raw in Southeast Asia. In Indonesia, they are steamed and eaten as a vegetable with salted fish and rice, and also used as seasoning for various dishes. They are sometimes cooked with meat to tenderize it.

Food Value per 100 g of Edible Portion

Table 1.3: Food value/100g

Calories	157.30
Total Solids	14.53-40-35%
Moisture	59.65-85.47%
Protein	0.50-0.80%
Fat	0.28-1.79%
Sugar (sucrose)	8.05-10-54%
Acid	0.47%
Crude Fiber	0.85-3-60%
Ash	0.44-0.65%

Figure 1.2: Spondias pinnata plant

Figure 1.3: Spondias pinnata fruit

1.4.5. Medicinal Uses of *Spondias pinnata*

Plant Part	Medicinal properties
Leaf	It is aromatic, acidic, appetizing and astringent, and is used in dysentery. The juice of the leaves is recommended for local application in otalgia (Ethnobotanybd, 2010). In Nigeria, a decoction of the mashed leaves is used for washing a swollen face. A leaf infusion is a common cough remedy & used as a laxative for fever with constipation. A leaf decoction is used for gonorrhoea. All these leaves are used for leprosy. Crushed with lemon they are effective for worms in children. A decoction of pounded leaves is used as an eye lotion and the juice pressed from young, warm leaves is given to children for stomach troubles. The young leaves are used as an infusion taken internally or as a warm astringent lotion by women in confinement in Sierra Leone. In Suriname's traditional medicine, the infusion of the leaves is used as a treatment of eye inflammation, diarrhoea and venereal diseases (Faiz M, 2011).
Fruit (Unripe)	It is astringent, sour, thermogenic, appetizer and aphrodisiac, and is good for rheumatism and sore throat (Ethnobotanybd, 2010).
Root	Regulation of menstruation. It is used in fever in Thailand (Faiz M, 2011).
Fruit (Ripe)	It is sweet, astringent, cooling, emollient, tonic, constipating and antiscorbutic, and is administered in bilious dyspepsia, diarrhea, and vitiated conditions of tridosa. Ripe fruit is aphrodisiac & cures burning sensation. The fruit-juice is used as a febrifuge and diuretic (Ethnobotanybd, 2010).
Bark	It is aromatic, astringent and refrigerant, and infusion of the bark is administered in dysentery, diarrhea, vomiting. Paste of the bark is used as an embrocation for both articular and muscular rheumatism. Decoction of the bark is given in gonorrhoea & severe cough. Gum of the bark is demulcent. Bark is used as purgative and in local applications for leprosy (Faiz M, 2011).

In Cambodia, the bark of *Spondias pinnata* is used with various species of *Terminalia* as a remedy for diarrhea.

1.4.6 Reported Biological Works on *Spondias pinnata*:

Plant part	Investigation & Result	Reference
Whole plant	The ethanolic extract was investigated for total phenolic activity, total flavonoid & free radical scavenging activity.	Maisuthisakul, P. *et al* (2007)
Fruit	The methanolic extract was investigated for total phenolic activity, total flavonoid & DPPH radical scavenging activity.	Wetwitayaklung, P. *et al* (2012)
Stem bark	The 70% methanolic extract was investigated for hydroxyl radical scavenging, superoxide radical scavenging, NO radical scavenging, Hydrogen peroxide radical scavenging, Peroxynitrite radical scavenging, Singlet oxygen scavenging, Hypochlorous acid scavenging, reducing power, ferrous chelation, total phenolic activity & total flavonoid activity.	Hazra, B. *et al* (2008)
Fruit	The extract was investigated for total phenolic activity, total flavonoid & DPPH radical scavenging & ferric reducing activity.	Kubola, J. *et al* (2011)
Stem heart wood	The methanolic and ethyl acetate extract	Rao, BG. *et al* (2010).

	was investigated for hepatoprotective activity.	
Bark	Hypoglycemic activity of the bark of *Spondias pinnata* Linn. kurz	Mondal, S. *et al.* (2009)
Whole plant	Isolation of 24-methylelle cydoartanone, stigma-4en-3one, lignoceric acid, β-sitosterol and its β- D-glucoside	Rastogi, R.P. *et al* (1976)

Chapter-2: Collection and preparation of the plant material

2.1. Collection

Spondias pinnata fruit was collected from Dhaka in August 2011. The plant was identified by Bangladesh National Herbarium. One voucher specimen has been deposited in Bangladesh National Herbarium (DACB accession no. 36703) and another one to East West University. Skin of the fruit was separated from the fruit and then air dried for several days. The dried skin was then grounded in course powder in Holy Chemical Lab (Tongi.) using high capacity grinding machine.

2.2 Extraction of the Plant material

About 500 gm of the powdered material was taken in separate clean, container (2.5 liters) and soaked in 2 liters of ethanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 55°C with a rotary evaporator (IKA RRV05 Basic, Biometra).

Figure 2.1: Rotary evaporator

The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained was 11gm.

2.3. Solvent-Solvent Partition of Crude extract

2.3.1. Preparation of Mother Solution

5 gm of ethanolic extract was triturated with 90 ml of ethanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned off successively by three solvents of different polarity. In subsequent stages each of the fractions was analyzed separately for the detection of any biological properties.

2.3.2. Partitioning with n-Hexane

The mother solution was taken in a separating funnel. 100 ml of the *n*-hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice; *n*-hexane fractions were collected together evaporated in Rota evaporator.

2.3.3. Partitioning with Dichloromethane

To the mother solution left after washing with *n*-hexane, 12.5 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with Dichloromethane (100 ml X 3). The Dichloromethane fractions were collected together and evaporated. The aqueous fraction was preserved for the next step.

2.3.4. Partitioning with Ethyl acetate

To the mother solution that left after washing with *n*-hexane and Dichloromethane, 16 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with Ethyl acetate (100 ml X 3). The $CHCl_3$ soluble fractions were collected together and evaporated. The aqueous ethanolic fraction was preserved as aqueous fraction.

Figure 2.2: Solvent-Solvent Partition of Crude extract

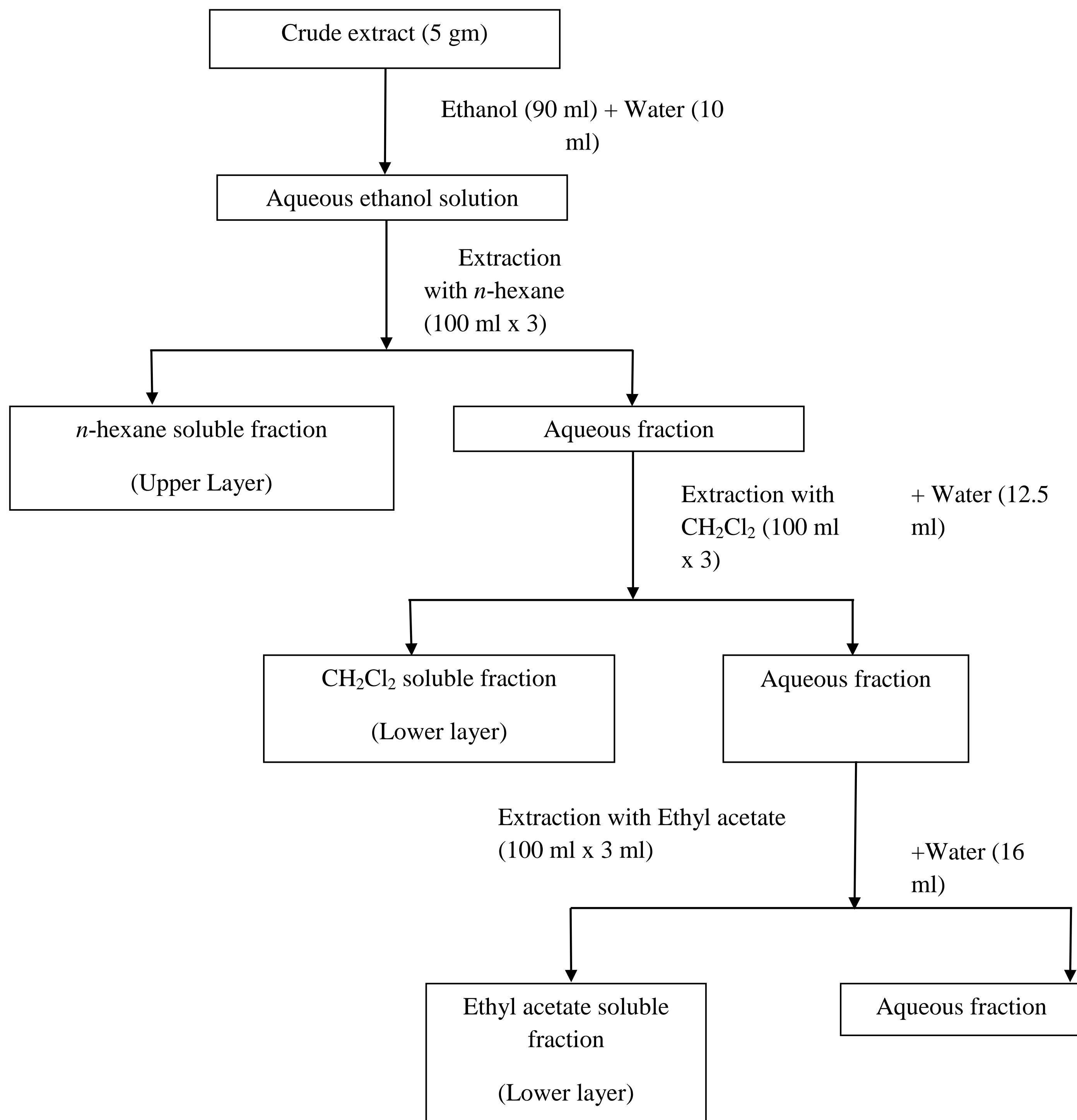


Figure 2.3: Schematic representation of the liquid-liquid extraction of ethanolic crude extract of *Spondias pinnata*

After evaporation the weight of the different fractions obtained are as follows:

Table 2.1: Weight of different fraction.

Plant	**Part**	**Fraction**	**Weight**
Spondias pinnata	**Fruit skin**	n-hexane soluble fraction	700 mg
		Carbontetrachloride soluble fraction	350 mg
		Chloroform soluble fraction	800 mg

Chapter 3: Design of *in vivo* investigations of the *S. pinnata*

3.1. General Approaches to Drug Discovery from Natural Sources

New medicines have been discovered with traditional, empirical and molecular approaches. The traditional approach makes use of drug that has been found by trial and error over many years in different cultures and systems of medicine. Examples include drugs like morphine, quinine and ephedrine that have been in widespread use for a long time, and more recently adopted compounds such as the antimalarial artemisinin. (Md. Shahidullah, 2009)

The empirical approach builds on an understanding of a relevant physiological process and often develops a therapeutic agent from a naturally occurring lead molecule. Examples include tubocurarine and other muscle relaxants, propranolol and other β-adrenoceptor antagonists, and cimetidine and other H2 receptor blockers. The molecular approach is based on the availability or understanding of a molecular target for the medicinal agent. With the development of molecular biological techniques and the advances in genomics, the majority of drug discovery is currently based on the molecular approach. The major advantage of natural products for random screening is the structural diversity. Bioactive natural products often occur as a part of a family of related molecules so that it is possible to isolate a number of homologues and obtain structure-activity relationship. Of course, lead compounds found from screening of natural products can be optimized by traditional medicinal chemistry or by application of combinatorial approaches. Overall, when faced with molecular targets in screening assays for which there is no information about low molecular weight leads, use of a natural products library seems more likely to provide the chemical diversity to yield a hit than a library of similar numbers of compounds made by combinatorial synthesis. Since only a small fraction of the world's biodiversity has been tested for biological activity, it can be assumed that natural products will continue to offer novel leads for novel therapeutic agents. That is why it is important to do research on the plant to discover the new window of new medicine which will help us to fight against the new diseases.

3.2. Experimental Design

3.2.1. Preliminary Phytochemical Screening

The crude extract of the plant was tested to determine the presence of carbohydrates, tannins, steroids, saponins, alkaloids, flavonoids, reducing sugars.

3.2.2. Microbiological Investigations

The *in vitro* antimicrobial study was designed to investigate the antibacterial as well as antifungal spectrum of the crude extracts and fractions by observing the growth response. The rationale for these experiments is based on the fact that bacteria and fungi are responsible for many infectious diseases, and if the test materials inhibit bacterial or fungal growth then they may be used in those particular diseases.

However, a number of factors viz. the extraction method (Nadir *et al.*, 1986), inocula volume, culture medium composition (Bauer *et al.*, 1966), pH (Levan *et al.*, 1979), and incubation temperature (Lorian, 1991) can influence the results.

Antimicrobial activity was observed by using two methods. The methods are-

a. Kirby- Bauer disk diffusion method.
b. Determination of Minimum Inhibitory Conentration (MIC) & Minimum Bactericidal Concentration (MBC).

3.2.3. Antioxidant test

Antioxidant test was done to determine the antioxidant capacity of the various fraction of the *S .pinnata* skin. The traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. Experiment evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a high number of diseases. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity. Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts.

Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans. Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant.

Antioxidant property of the various fraction of the plant was determined by following methods-

- Determination of total phenolic content
- Determination of DPPH radical scavenging assay
- Determination of reducing power ability
- Phosphomolbdate method
- Determination of total flavonoid content

3.2.4. Thrombolytic activity test

One of the major causes of blood circulation problem is the formation of blood clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area. A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). Fribrinolytic drugs has been used to dissolve thrombi in acutely occluded coronary arteries there by to restore blood supply to ischaemic myocardium, to limit necrosis and to improve prognosis

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs. The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed. Therefore much current research devoted to the phytochemical investigation of higher plants which have ethnobotanical information associated with them. The phytochemicals isolated are then screened for different

types of biological activity like thrombolytic potentials. Herbal preparations are used potential source of medicine since ancient times to maintain health and regain healthy state of mind. Herbs showing thrombolytic activity have been studied and some significant observations have been reported.

Chapter-4: Preliminary Phytochemical Screening

4.1. Introduction

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents using the following reagents and chemicals:

1. Alkaloids with Dragendorff's reagent,
2. Flavonoids with the use of Mg and HCl;
3. Tannins with ferric chloride and potassium dichromate solutions and
4. Saponins with ability to produce stable foam and steroids with Libermann Burchard reagent,
5. Reducing sugars with Benedict's reagent.

4.2. Procedures

1. **Test for Carbohydrates** (S. De, 2010)

Benedict's test

Treat the test solution with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath, reddish brown precipitate forms if reducing sugars are present.

Fehling's test

Equal volume of Fehling's A (Copper sulfate in distilled water) and Fehling's B (Potassium tartarate and Sodium hydroxide in distilled water) reagents are mixed and few drops of sample is added and Boiled, a brick red precipitate of cuprous oxide forms, if reducing sugars are present.

2. **Test for Sterols & Triterpenoids**

Libermann- Buchard test

Extract is treated with few drops of acetic anhydride, boil and cool, con. Sulfuric acid is added from the sides of the test tube, shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of Steroids

3. **Test for Alkaloid** (**Dragendorff's test)**

Alkaloids give reddish brown precipitate with Dragendorff's reagent [Potassium bismuth iodide solution]

4. **Test for Cardiac Glycosides**

Keller killiani test [test for Deoxy sugars]

Extract the drug with chloroform and evaporate it to dryness. Add 0.4ml of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5ml of

concentrated sulphuric acid by the side of the test tube, blue color appears in the acetic acid layer.

5. **Test for Tannins & Phenolic Compounds**

Ferric chloride test

Test solution gives blue green color with ferric chloride.

6. **Test for Flavonoids**

Alkaline reagent test

To the test solution add few drops of sodium hydroxide solution; formation of an intense yellow color, which turns to Colorless on addition of few drops of dil. acid, indicates presence of Flavonoids.

4.3. Results:

Table 4.1: Results of preliminary phytochemical test

Extract	Carbohydrate	Saponin	Steroid	Alkaloid	Cardiac glycoside	Tanin	Flavanoid
n-hexane fraction of *S. pinnata* skin	-	-	+	+	+	+	+
Dichloromethane fraction of *S. pinnata* skin	-	-	+	+	+	+	+
Ethyl acetate fraction of *S. pinnata* skin	-	-	+	+	+	+	+
Aquous fraction of *S. pinnata* skin	-	-	-	+	-	+	+

4.4. Discussion:

All of the fractions have shown the presence of the alkaloid,flavonoid and tannins in all tests. No fraction has shown the presence of the carbohydrates and saponin.

There is an evidence of the presence of cardiac glycoside in all fractions except aqueous fraction.

Positive results in the test of steroid showing the presence steroid in all fractions except aqueous fraction.

Chapter-5: Antimicrobial screening

5.1. Determination of Antimicrobial activity by Disc Diffusion Method

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States.

Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992; an increase of 58% .It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US (Pinner *et al*., 1996). This is alarming given that it was once believed that we would eliminate infectious disease by the end of the millenium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–44 year old age group (Pinner *et al*., 1996).

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials (Fauci, 1998).

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by disc diffusion method.

But there is no standardized method for expressing the results of antimicrobial screening (Ayafor *et al*., 1982). Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition (Bayer *et al*., 1966), p^H, and incubation temperature can influence the results.

Among the above mentioned techniques the disc diffusion (Bayer *et al*., 1966) is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity

or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method (Roland R, 1982).

5.2. Principle of Disc Diffusion Method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms.

Standard antibiotic discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media (Barry, 1976).

The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry, 1976; Bayer *et al.,* 1966.) In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bayer *et al.,* 1966).

5.3. Experimental

5.3.1. Apparatus and Reagents

1. Filter paper discs
2. Autoclave
3. Nutrient Agar Medium
4. Laminar air flow hood
5. Petridishes
6. Spirit burner
7. Sterile cotton
8. Refrigerator
9. Micropipette
10. Incubator
11. Inoculating loop
12. Ethanol
13. Sterile forceps
14. Nosemask and Hand gloves
15. Screw cap test tubes

5.3.2. Test Materials of Spondias pinnata

1. *n*-hexane soluble fraction of ethanolic extract
2. CH_2Cl_2 soluble fraction of ethanolic extract
3. Ethyl acetate soluble fraction of ethanolic extract
4. Aqueous soluble fraction of the ethanolic extract

5.3.3. Test Organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the East West University microbiology lab. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 5.1.

Table 5.1: List of micro-organisms used

Gram positive Bacteria	**Gram negative Bacteria**	**Fungi**
Bacillus cereus	*Escherichia coli*	*Candida albicans*
Bacillus subtilis	*Salmonella paratyphi*	*Sacharomyces cerevacae*
Sarcina lutea	*Salmonella typhi*	
Staphylococcus aureus	*Shigella boydii*	
	Shigella dysentery	
	Pseudomonas aeruginosa	
	Vibrio mimicus	

5.3.4. Culture Medium and their Composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

a. Nutrient agar medium

Ingredients	Amount
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml

Agar medium having this composition was directly brought from the market.

5.3.5. Preparation of the Medium

To prepare required volume of this medium, calculated amount of agar medium was taken in a bottle with a cap and distilled water was added to it to make the required volume. The contents were then autoclaved to make a clear solution.

5.3.6. Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Figure 5.1: Laminar hood

5.3.7. Preparation of Subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

Figure 5.2: Incubator

5.3.8. Preparation of the Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium.

The bacterial and fungal suspension was taken by a loop a mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial/ fungal suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud.

5.3.9. Preparation of Discs

Three types of discs were used for antimicrobial screening.

5.3.10. Standard Discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Cephradin (30µg/disc) standard disc was used as the reference.

5.3.11. Blank Discs

These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

5.3.12. Preparation of Sample Discs with Test Sample

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition.

Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

5.3.13. Preparation of Sample Discs with Test Sample

Measured amount of each test sample (specified in table) was dissolved in specific volume of solvent (chloroform or methanol) to obtain the desired concentrations in an aseptic condition.

Table 5.2: Preparation of Sample Discs

Plant	**Sample**	**Dose** (µg/disc)	**Required amount for 20 disc** (mg)
Spondias pinnata	Hexane soluble fraction of ethanolic extract	400	8.0
	Hexane soluble fraction of ethanolic extract	800	16
	CH_2Cl_2 soluble fraction of ethanolic extract	400	8.0
	CH_2Cl_2 soluble fraction of ethanolic extract	800	16
	Ethyl acetate soluble fraction of ethanolic extract	400	8.0
	Ethyl acetate soluble fraction of ethanolic extract	800	16
	Aqueous soluble fraction of ethanolic extract	400	8.0
	Aqueous soluble fraction of ethanolic extract	800	16

5.3.14. Application of the Test Samples

Standard Cephradin (30 μg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

5.3.15. Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

5.3.16. Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

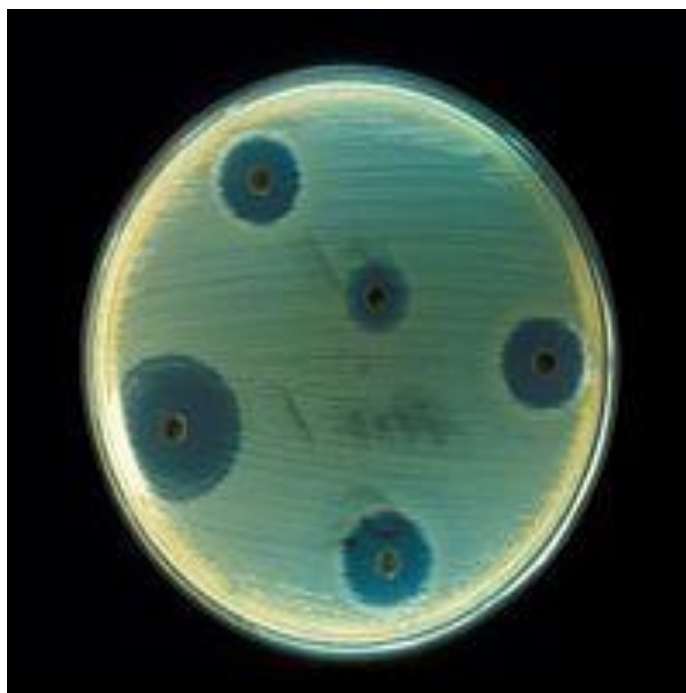


Figure 5.3: Zone of inhibition

5.4. Result & Discussion

5.4.1. In vitro antimicrobial screening

Table 5.3: Results of antimicrobial test

Microorganisms	**Crude 800**	**Crude 400**	**SPSNH 800**	**SPSNH 400**	**SPSDM 800**	**SPSDM 400**	**SPSEA 800**	**SPSEA 400**	**SPSAQ 800**	**SPSAQ400**	**Control**
Shigella boydii	20	15	13	10	18	15	0	0	7	0	23
Bacillus subtilis	18	16	18	15	15	7	0	0	0	0	26
Candida albicans	16	15	9	0	15	12	0	0	0	0	27
Saccharomyces cerevisiae	18	14	16	12	14	12	0	0	0	0	20
Vibrio mimicus	15	10	7	0	16	11	0	0	0	0	22
Shigella dysentery	20	14	0	0	17	14	18	13	12	11	22
Escherichia coli	16	14	7	7	13	12	0	0	0	0	27
Bacillus cereus	18	16	7	0	15	11	7	0	0	0	25
Salmonella typhi	15	8	8	7	13	11	7	0	0	0	20
Salmonella paratyphi	12	8	7	7	10	7	0	0	0	0	25
Pseudomonas aeruginosa	25	18	11	10	13	11	22	17	16	12	25
S.lutea	15	13	0	0	11	9	0	0	0	0	21
Staphylococcus aureus	22	15	0	0	19	15	0	0	0	0	15

The antimicrobial activities of extracts were examined in the present study. The crude extract produced strong activity against most of the organisms that's why fraction of the crude extract was subjected to the antimicrobial test. The results are given in table. The zones of inhibition produced by the *n*-hexane, dichloromethane, ethyl acetate and aqueous soluble partitionates of the ehanolic extract were ranged from 0-15 mm, 7-14 mm, 0-17 mm and 0-12 mm respectively at a concentration of 400µg/disc.

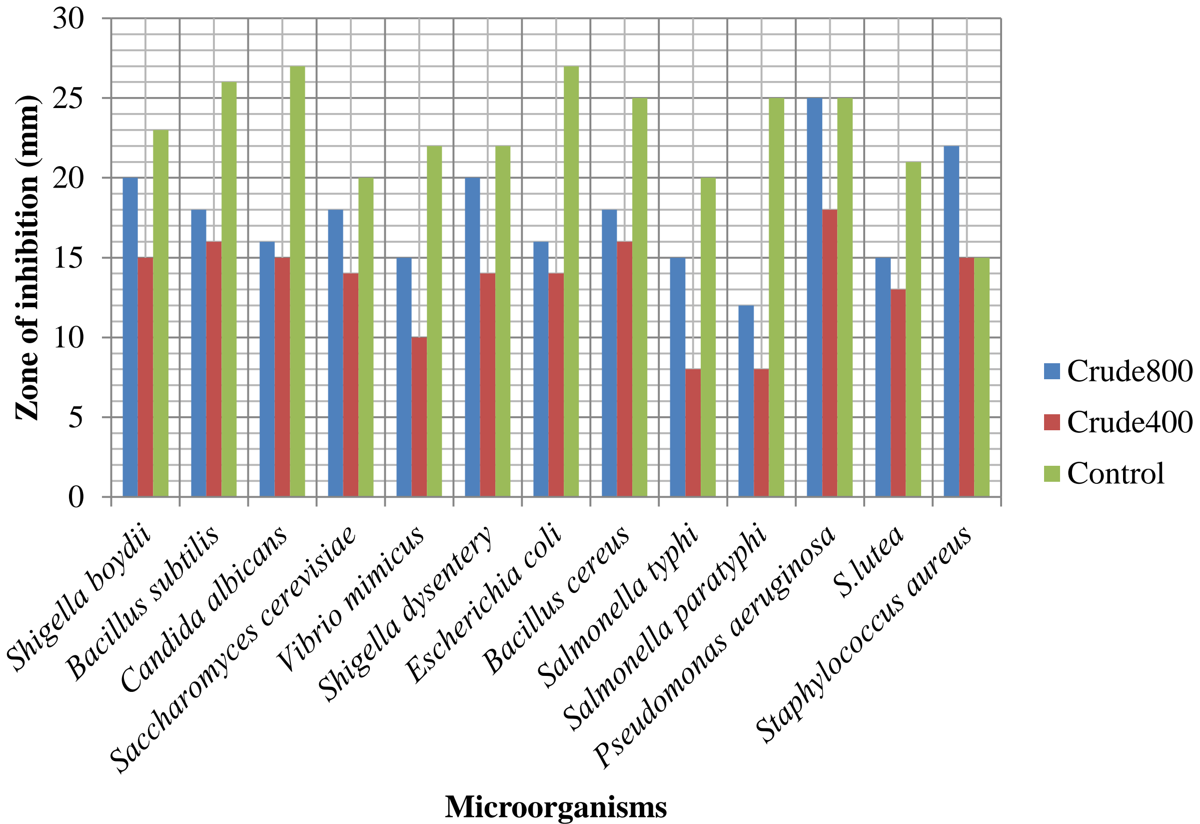


Figure 5.4: Comparison of the zone of inhibition of crude extract with the control

The zones of inhibition produced by the *n*-hexane, dichloromethane, ethyl acetate and aqueous soluble partitionates of the ehanolic extract were ranged from 0-13 mm, 10-19 mm, 0-18 mm and 0-16 mm respectively at a concentration of 800µg/disc.

The n-hexane partitionate of the ehanolic extract showed the strongest activity against *Bacillus subtilis* having the zone of inhibition of 15 mm (400µg) and 18 mm (800 µg). Moderate activity was found against *Shigella boydii, S. typhi, S. paratyphi*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *E.coli*. Weak activity observed against *Vibrio mimicus.*

No activity is shown against *S.lutea, Shigella dysentery, Staphylococcus aureus*

Among the fungi, the *S. cerevaceae* (16mm for 800µg and 12mm for 400µg) was also strongly inhibited. The growth of *C. albicans* was weakly inhibited having zone size 0 mm amd 9mm for 400µg and 800µg respectively.

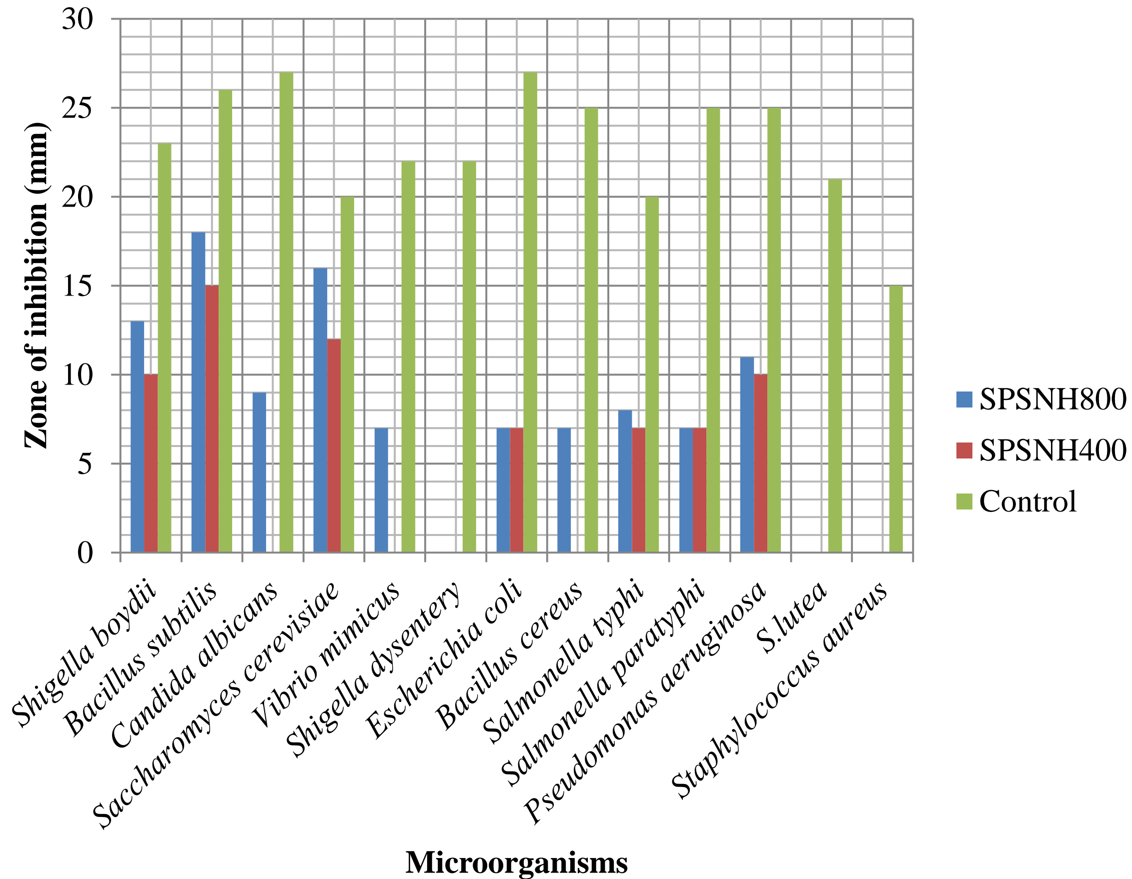


Figure 5.5: Comparison of the zone of inhibition of n-hexane soluble fraction with the control

The dichloromethane partitionate of the ehanolic extract showed strongest activity against *Shigella boydii* (18mm for 800µg and 15 mm for 400µg) and *S. aureus* (19mm for 800µg and 15mm for 400µg). The growth of *Bacillus subtilis* (15 mm for 800µg and 7mm for 400µg), *B. cereus* (15mm for 800µg and 11mm for 400µg), *Shigella dysentery* (17mm for 800µg and 14mm for 400µg) *and Vibrio mimicus* (16mm for 800µg and 11mm for 400µg) were strongly inhibited. Moderate activity was found against *E. coli* (13mm for 800µg and 12 mm for 400µg), *Salmonella typhi* (13mm for 800µg and 11 mm for 400µg), *Salmonella paratyphi* (10mm for 800µg and 7 mm for 400µg), *Pseudomonas aeruginosa* (13mm for 800µg and 11 mm for 400µg), *S.lutea* (11mm for 800µg and 9 mm for 400µg).

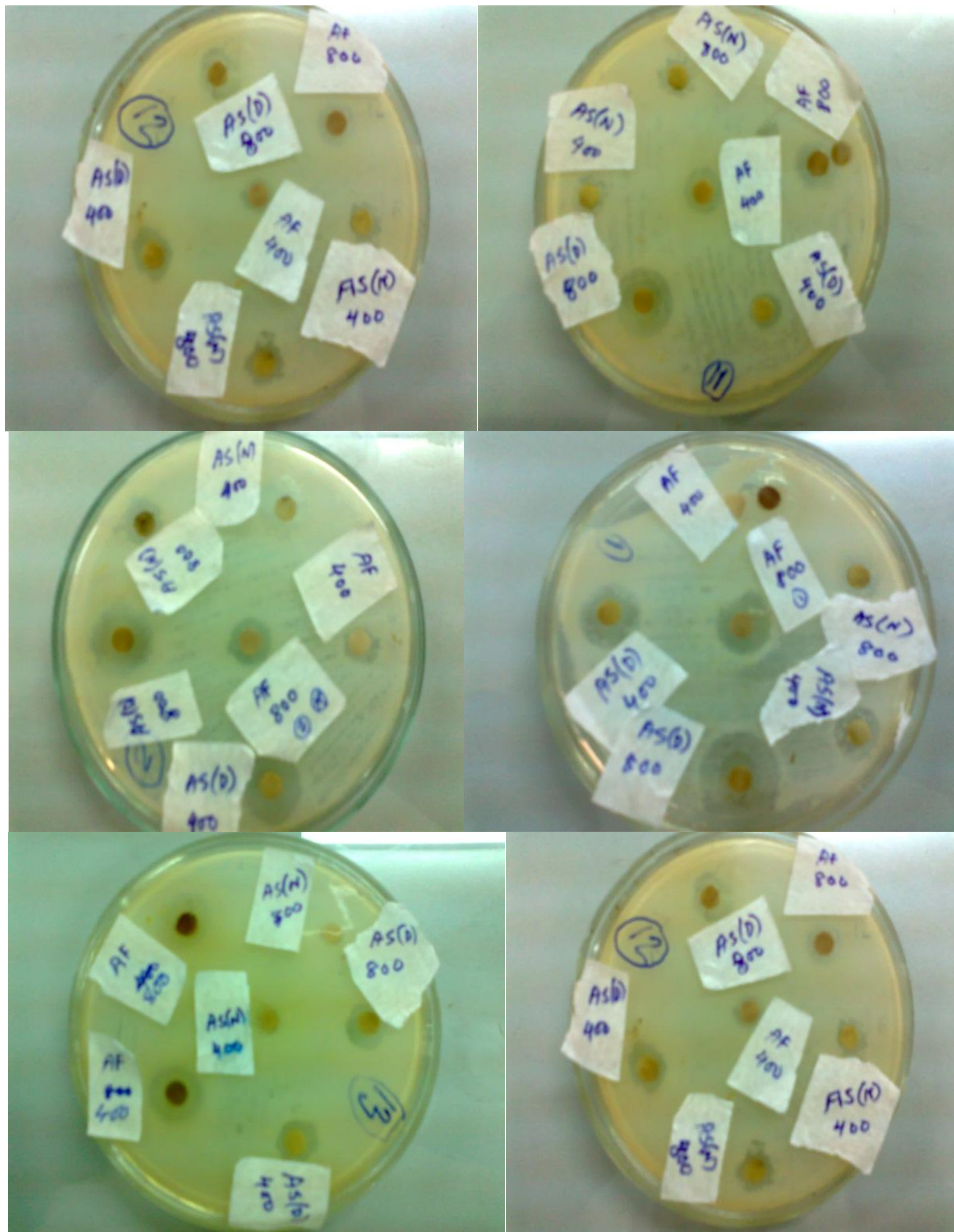


Figure 5.6: Antimicrobial test for *S. pinnata* skin

In case of fungi, the growth of *C. albicans* (14mm for 800µg and 12mm for 400µg) and *S. cerevaceae* (15mm for 800µg and 12 mm for 400µg) strongly inhibited.

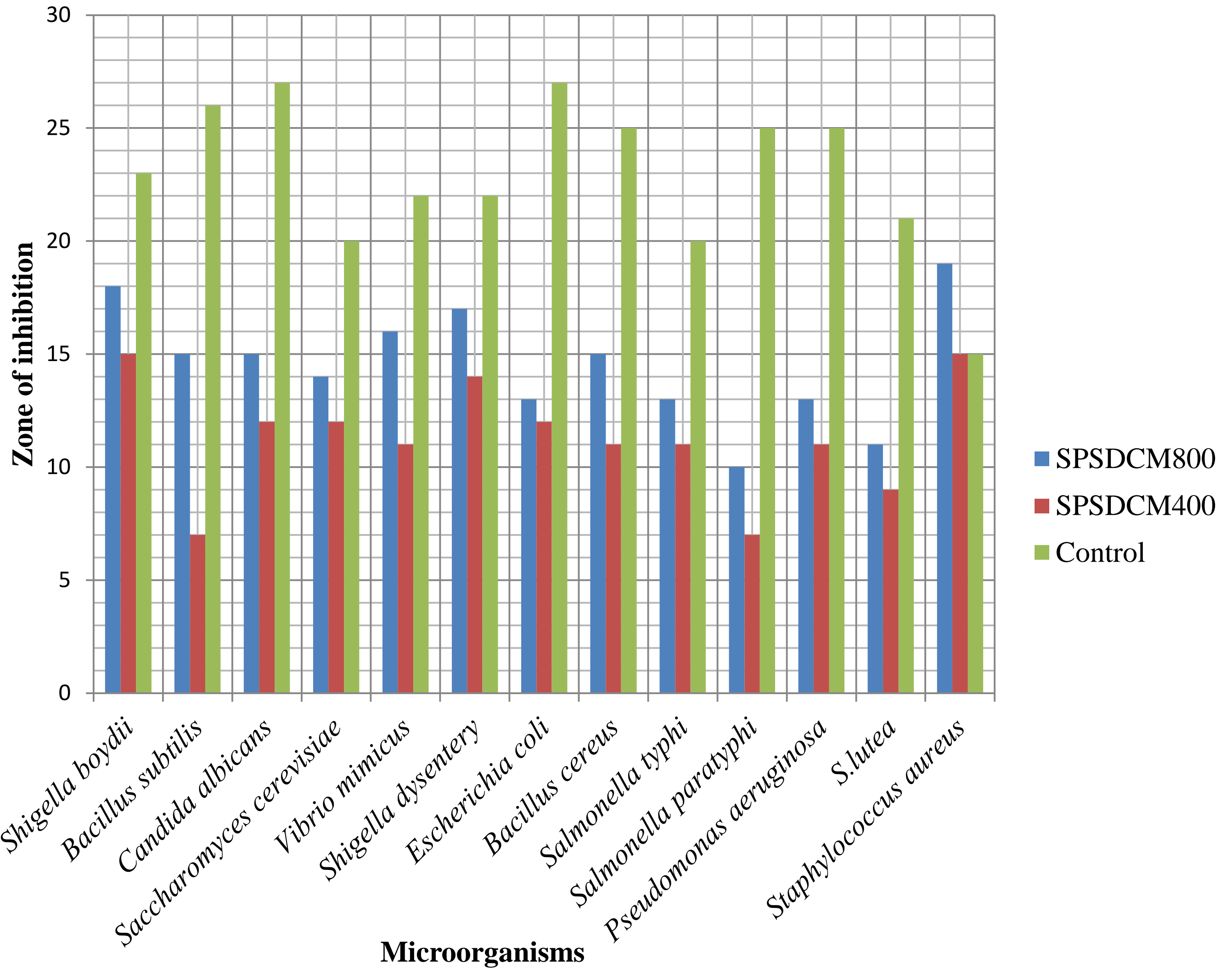


Figure 5.7: Comparison of the zone of inhibition of dichloromethane soluble fraction with the control

The ethyl acetate partitonate of the ethanolic extract showed strongest activity against *Shigella dysentery*(18mm for 800µg and 13 mm for 400µg), *Pseudomonas aeruginosa* (22mm for 800µg and 17 mm for 400µg)

Weak activity has been shown against *Bacillus cereus* (7mm for 800µg and 0 mm for 400µg), *Salmonella typhi* (7mm for 800µg and 0 mm for 400µg) and no activity has been shown against other organisms.

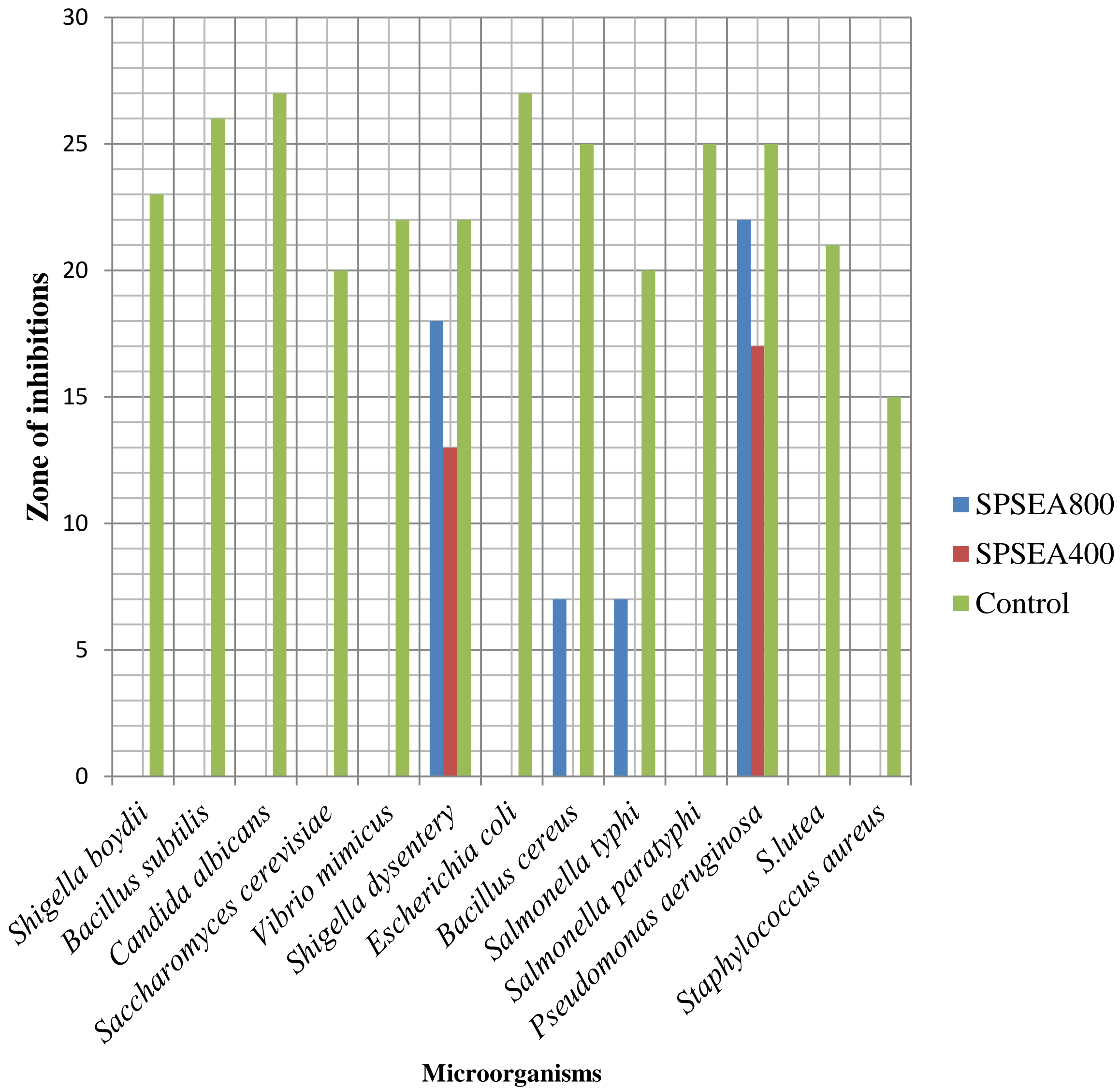


Figure 5.8: Comparison of the zone of inhibition of ethyl acetate soluble fraction with the control

The aqueous partitionate of the ehanolic extract showed moderate to strong activity against the tested microorganisms. The growth of *Pseudomonas aeruginosa* (16mm for 800µg and 12 mm for 400µg) was strongly inhibited. It also showed moderate activity against *Shigella dysentery*

(12mm for 800µg and 11 mm for 400µg) and weak activity has been shown for *Shigella boydii* (7mm for 800µg and 0 mm for 400µg). No activity has been shoen for other organisms.

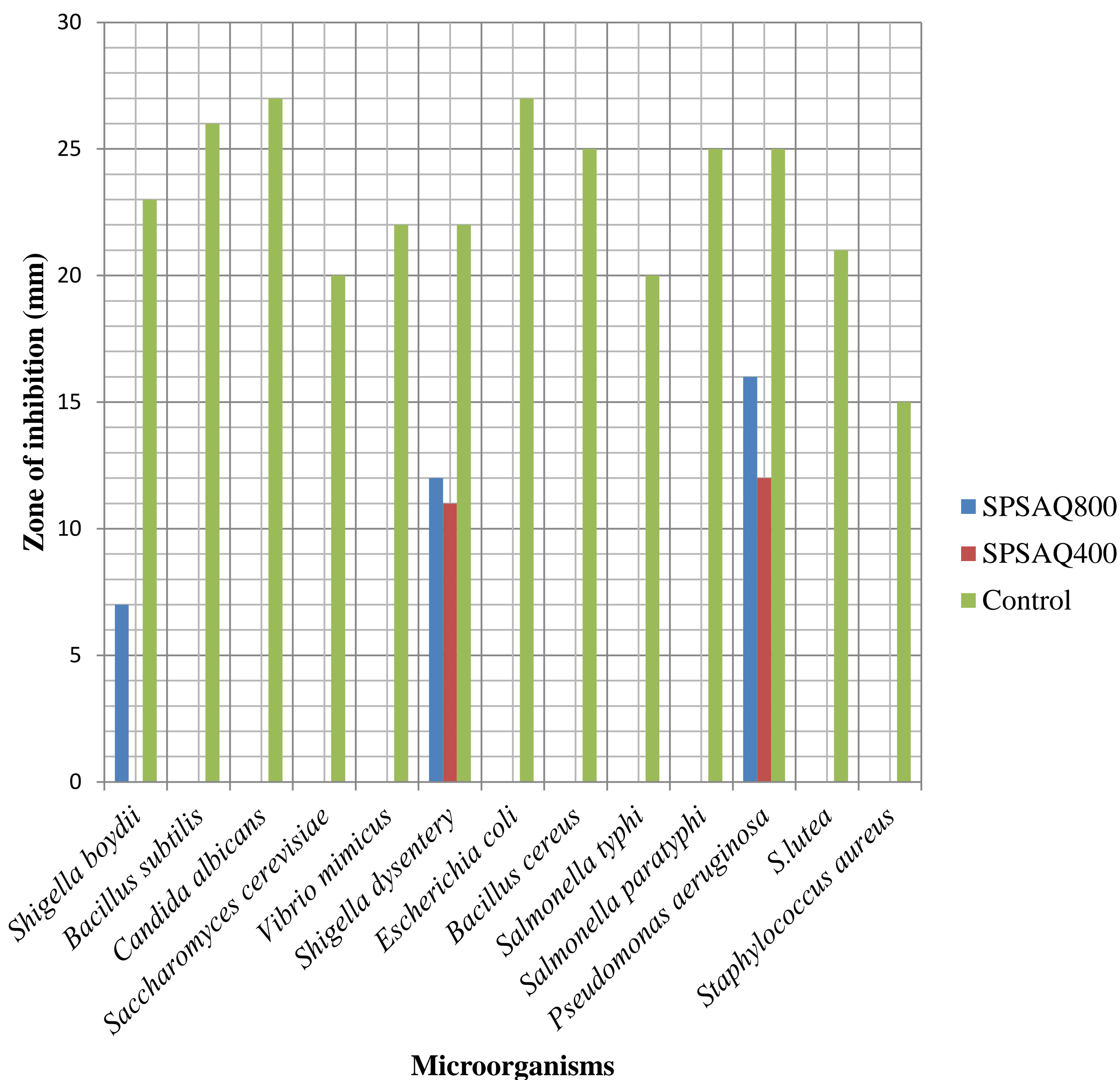


Figure 5.9: Comparison of the zone of inhibition of aqueous fraction with the control

Out of all the samples, dichloromethane soluble partitionates of the ehanolic extract were appeared very potent in terms of both zone of inhibition & spectrum of activity. Bioactivity guided isolation can be carried out to separate there bioactive metabolites.

5.5. Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration)

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the fraction ethanolic extract required to kill *S. aureus, S. typhi, S. paratyphi and S. boydi*. In the experiment, medicaments were added to bacterial species into eppendrof tube, in 10 different concentrations. The MIC was the lowest concentration of the drug at which bacterial growth could not be observed.

5.6 Principle of MIC

The disc diffusion method, which is a 'semi-quantitative' method, determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. Next to the agar dilution method, the broth dilution method is one of the most frequently used methods to determine MIC's. The method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the extract for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined is then considered as the MIC.

5.7. Experimental

5.7.1. Apparatus and Reagents

Nutrient Agar Medium	Autoclave
Nutrient Broth Medium	Eppendrof tube
Petridishes	Laminar air flow hood
Sterile cotton	Spirit burner
Micropipette	Refrigerator
Inoculating loop	Incubator
Sterile forceps	Ethanol
Screw cap test tubes	Nosemask and Hand gloves

5.7.2. Test Materials of *Spondias pinnata*

- *n*-hexane soluble fraction of ethanolic extract
- CH_2Cl_2 soluble fraction of ethanolic extract
- Ethyl acetate soluble fraction of ethanolic extract
- Aqueous soluble fraction of the ethanolic extract

5.7.3. Test Organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the East West University microbiology lab. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 4.1.

Table 5.4: List of micro-organisms used

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus	*Salmonella paratyphi* *Salmonella typhi* *Shigella boydii*

5.7.4. Culture Medium and their Composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

b. Nutrient agar medium

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extracts	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
P^H	7.2 + 0.1 at 25^0C

Agar medium having this composition was directly brought from the market.

c. Nutrient broth medium

Ingredients	Amount
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.	100 ml
P^H	7.2 ±0.1 at 25^0C

5.8 Method description (Huys, 2002)

I. Bacterial cultivation and material preparations (DAY 1-2)

• The organism to be tested should be sub cultured using a suitable medium under optimal incubation conditions to obtain a fresh overnight grown culture. As standard conditions, growth in Agar and Broth at 28 °C under aerobic atmosphere is recommended. For sub culturing, remove one bead from the cryovial and streak out onto an Agar plate. Incubate at 28°C overnight (or longer until clear visible growth is observed).

• After overnight incubation, the streak cultures are checked for purity. A number of pure colonies (app. 5 or more if the isolate is a suspected fastidious organism) are introduced into a glass culture tube containing 10 mL broth and incubated at 28°C overnight (or longer until clear visible growth is observed). Ideally, the culture tubes should fit into a portable spectrophotometer. In this way, the overnight grown culture can be easily adjusted to a standardized cell density by dilution with sterile broth

• Control cultures should be included during each series of MIC determinations.

• A bottle with the necessary volume of double-distilled water should be prepared.

II. Preparation and inoculation of the dilution series (DAY 3)

• The optical density of the overnight culture of the strain is determined spectrophotometrically at 590 nm and is standardized at 0.1 + 0.02 (i.e. app. 10E8 CFU/mL) by diluting with sterile broth.

• For each batch of 4 strains, two 6 mL sterile stock solutions of extract should be prepared in the suitable solvent. However, subsequent dilutions of these stock solutions can be made up in sterile water. The use of two stock solutions is recommended to minimize quantitative errors in the low-concentration range of the serial dilution series.

• Extract concentrations 40, 20, 10, 5, 2.5, and 1.25, 0.625, 0.3125, 0.15625, 0.078125 mg/ml are obtained by making serial dilutions from stock solution. It is absolutely crucial to thoroughly mix every freshly prepared antibiotic dilution prior to using it to prepare the next dilution. It should be noted that each antibiotic dilution undergoes a final 1:2 dilution when the broth culture is added. If required, the tested MIC range can be extended with additional concentrations.

• Following the preparation of the serial dilutions of extract, 2 mL of freshly standardized broth culture of the strain is inoculated in each tube of the dilution series. In this regard, it is important to note that the standardized cultures should be processed within the hour after preparation. Dilutions and broth cultures should be well homogenized prior to mixing.

• For each batch of MIC determinations, a blank tube (i.e. 2 mL non-inoculated broth mixed with 2 mL water) should be included. In addition, a positive control should be included for each strain. The positive control is made up by mixing 2 mL adjusted broth culture with 2 mL sterile double distilled water.

• All MIC tubes and control tubes of the test isolates and the control strain as well as the blanc are incubated aerobically at 28°C for 24h.

• Each isolate should also be checked for purity by plating a drop of the adjusted culture onto agar medium, and this plate should be incubated under the same conditions as the MIC test itself.

III. Reading of the MIC (DAY 4)

• The purity of the broth culture is checked on agar on the basis of uniform colonial morphology. If contamination is noted than all data generated from the involved strain should be rejected.

• Following a 24 h incubation (or longer until clear visual growth can be determined in the positive control tubes), growth is determined visually among the different tubes of the serial dilution by comparing with the positive control and with the blank. Any series where discontinuity in growth is observed (e.g. growth in tubes 5 and 7 but not in tube 6) should be discarded. The end-point is defined as the lowest antibiotic concentration for which there is no visual growth. This concentration should be reported as the MIC of that antibiotic for that particular strain. If trailing end-points are observed, this should be reported as a remark and a 80% reduction in growth should be reported as end-point.

Then a sterilized cotton bud is dipped into the clear solution obtained then applied to an agar plate.

IV. Reading of the MBC (DAY 5)

The agar plates then checked for the growth.

5.9. Results

Table 5.5: Result of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration determination test

Extract	**Bacteria**									
	(1)*S. aureus*		**(2)*S.paratyphi***		**(3) *S. typhi***		**(4)*B.subtilis***		**(5)*S.boydii***	
	MIC mg/ml	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**
n-hexane soluble fraction	>40	>40	20	>40	40	>40	>40	>40	>40	>40
CH_2Cl_2 fraction	2.5	5	0.625	2.5	0.625	1.25	0.078125	0.625	0.625	1.25
Ethyl acetate fraction	10	20	5	10	0.3125	1.25	0.3125	10	2.5	5
Aqueous soluble fraction	40	>40	40	20	2.5	10	40	>40	40	>40

5.10. Discussion

So, dichloromethane fraction produces comparatively good activity MIC ranging from 0.78125-2.5 mg/ml whereas MBC ranging from 1.25-5 mg/ml.

Figure 5.10: MIC test for dichloromethane fraction against *B. subtilus*

Figure 5.11: MIC test for dichloromethane fraction against *S. paratyphi*

Table 5.6: Result of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) for Dichloromethane fraction

Extract	Bacteria									
	(1)*S. aureus*		**(2)*S.paratyphi***		**(3) *S. typhi***		**(4)*B.subtilis***		**(5)*S.boydii***	
	MIC mg/ml	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**
CH_2Cl_2 fraction	2.5	5	0.625	2.5	0.625	1.25	0.078125	0.625	0.625	1.25

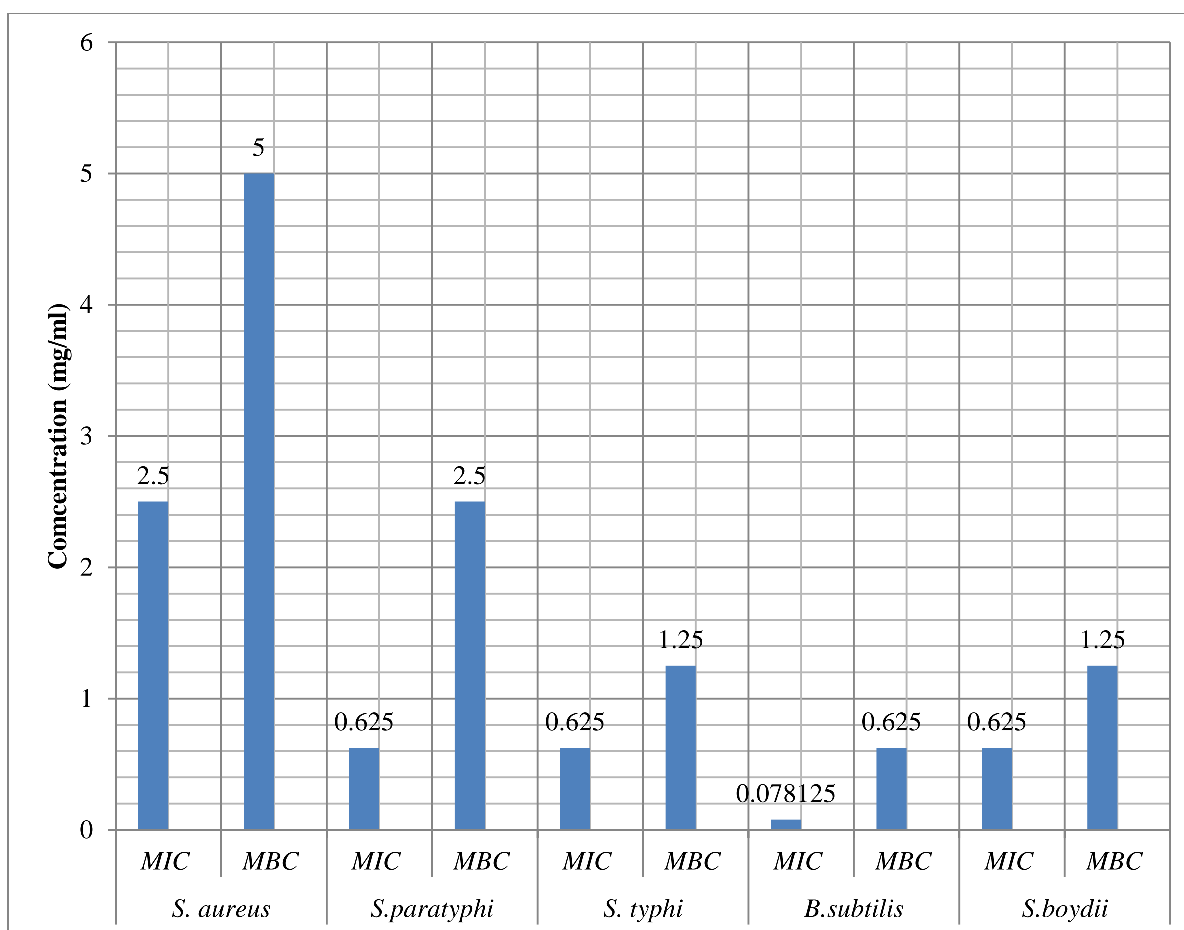


Figure 5.12: MIC and MBC for dichloromethane fraction

Figure 5.13: MIC test for dichloromethane fraction against *S. paratyphi*

Ethyl acetate fraction produces moderate activity MIC ranging from 0.3125-10 mg/ml and MBC ranging from 1.25-20mg/ml.

Table 5.7: Result of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) for Ethyl acetate fraction

Extract	**Bacteria**									
	(1)*S. aureus*		**(2)*S.paratyphi***		**(3) *S. typhi***		**(4)*B.subtilis***		**(5)*S.boydii***	
	MIC mg/ml	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**	**MIC mg/ml**	**MBC mg/ml**
Ethyl acetate fraction	10	20	5	10	0.3125	1.25	0.3125	10	2.5	5

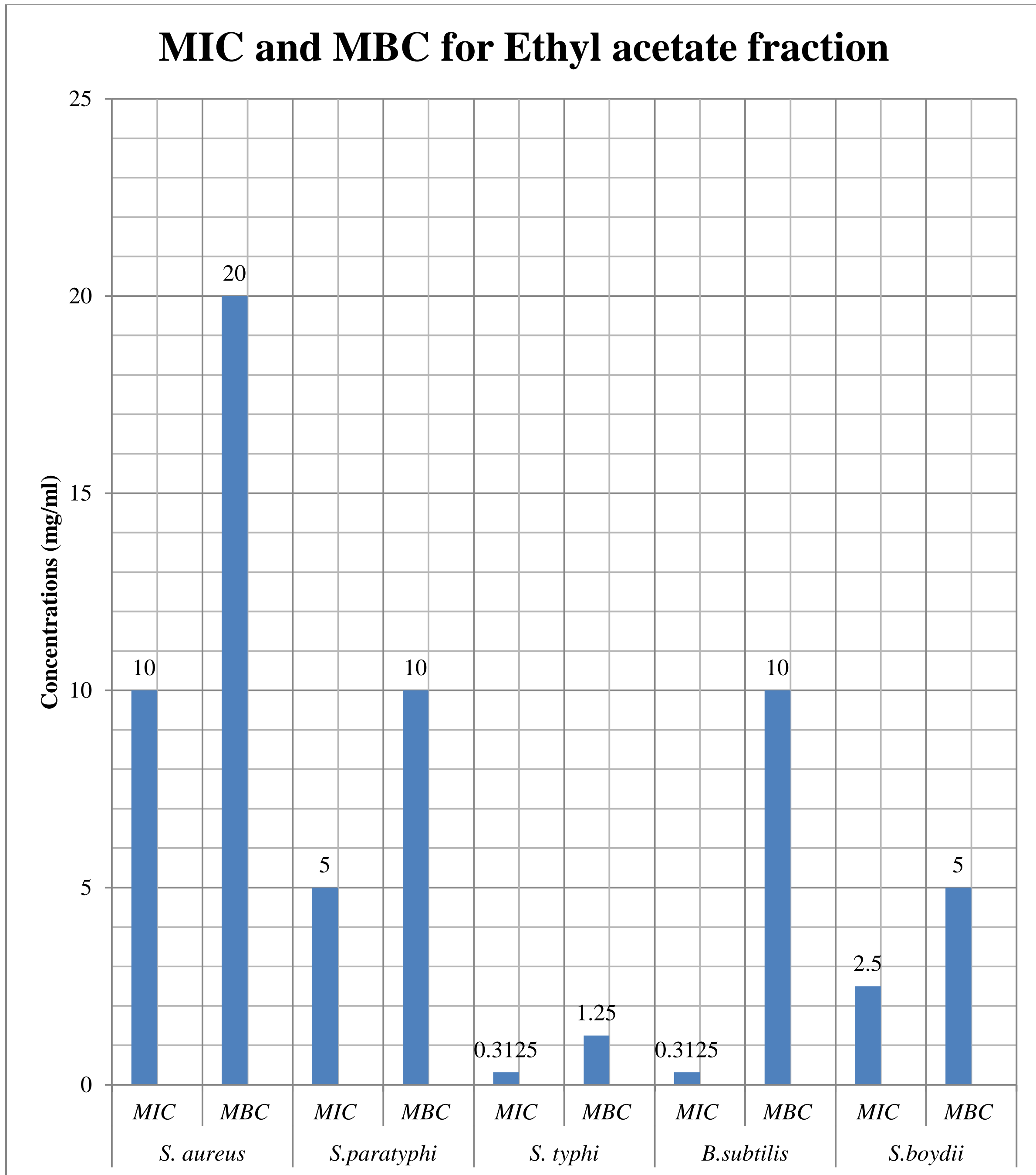


Figure 5.14: MIC and MBC for Ethyl acetate fraction

n-hexane soluble fraction and aqueous fraction produces weak activity.

Chapter-6: Antioxidant test

6.1. Rational and objective

There is considerable recent evidence that free radical induce oxidative damage to biomolecules. This damage causes cancer, aging, neurodegenerative diseases, atherosclerosis, malaria and several other pathological events in living organisms (Halliwell *et al.,* 1992). Antioxidants which scavenge free radicals are known to possess an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases (Steinmetz and Potter, 1996; Aruoma, 1998; Bandoniene *et al*., 2000; Pieroni *et al.,* 2002; Couladis *et al.,* 2003). A number of reports on the isolation and testing of plant derived antioxidants have been described during the past decade. Natural antioxidants constitute a broad range of substances including phenolic or nitrogen containing compounds and carotenoids (Shahidi *et al.,* 1992; Velioglu *et al.,* 1998; Pietta *et al.,* 1998)

Lipid peroxidation is one of the main reasons for deterioration of food products during processing and storage. Synthetic antioxidant such as *tert*-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) are widely used as food additives to increase self-life, especially lipid and lipid containing products by retarding the process of lipid peroxidation. However, BHT and BHA are known to have not only toxic and carcinogenic effects and humans (Ito *et al*., 1986; Wichi,1988), but abnormal effects on enzyme systems (Inatani *et al*., 1983). Therefore, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakasha & Jaganmohan Rao, 2000)

6.1.2 Methods of evaluating antioxidant activity

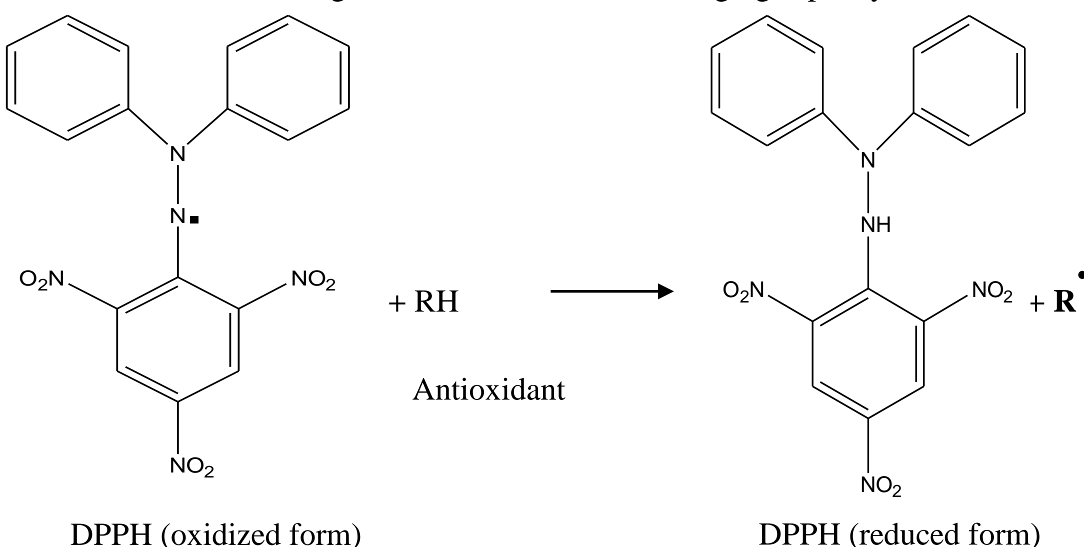
Antioxidant property of the various fraction of the plant was determined by following methods-

- Determination of DPPH radical scavenging assay (Quantitative analysis)
- Determination of total phenolic content
- Determination of reducing power ability
- Determination of total antioxidant capacity by phosphomolybdenum method
- Determination of total flavonoids content

6.1.1. Determination of DPPH radical scavenging assay (Quantitative analysis)

6.1.1.1. Principle

A rapid, simple and convenient method to measure free radical scavenging capacity of antioxidants involves the use of the free radical, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. DPPH is a stable nitrogen centered free radical with purple color and the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm. When the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, then the color turns from purple to yellow as the molar absorptivity of the DPPH radical reduces from 9660 to 1640 at 517 nm. Scavenging of DPPH free radicals by antioxidants decreases the absorbance. The lower the absorbance at 517 nm, the greater the free radical scavenging capacity of the crude extracts.



DPPH (oxidized form)

DPPH (reduced form)

Diphenyl picrylhydrazyl

6.1.1.2. Materials & Reagents

1,1-diphenyl-2-picrylhydrazyl
L-Ascorbic acid
Distilled water
Methanol
Pipette (5ml)
Analytical balance

UV- visible spectrophotometer
Beaker (100 & 200ml)
Test tube
Aluminium foil
Spatula

6.1.3. Methods

- 2.0 ml of a methanol solution of the extract at different concentration (2, 4, 6, 8, 10µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml).
- After 30 min reaction period at room temperature in dark place the absorbance was measured against at 517 nm against methanol as blank by using a UV- visible spetrophotometer.
- Inhibition free radical DPPH in percent (I%) was calculated as follows:
  (I%) = $(1 - A_{\textbf{sample}}/A_{\textbf{blank}})$ X 100

Where $A_{\textbf{blank}}$ is the absorbance of the control reaction (containing all reagents except the test material).

- Extract concentration providing 50% inhibition *(IC_{50})* was calculated from the graph plotted inhibition percentage against extract concentration.
- L-Ascorbic acid was used as positive control.
- Tests carried out in triplicate and average value was taken.

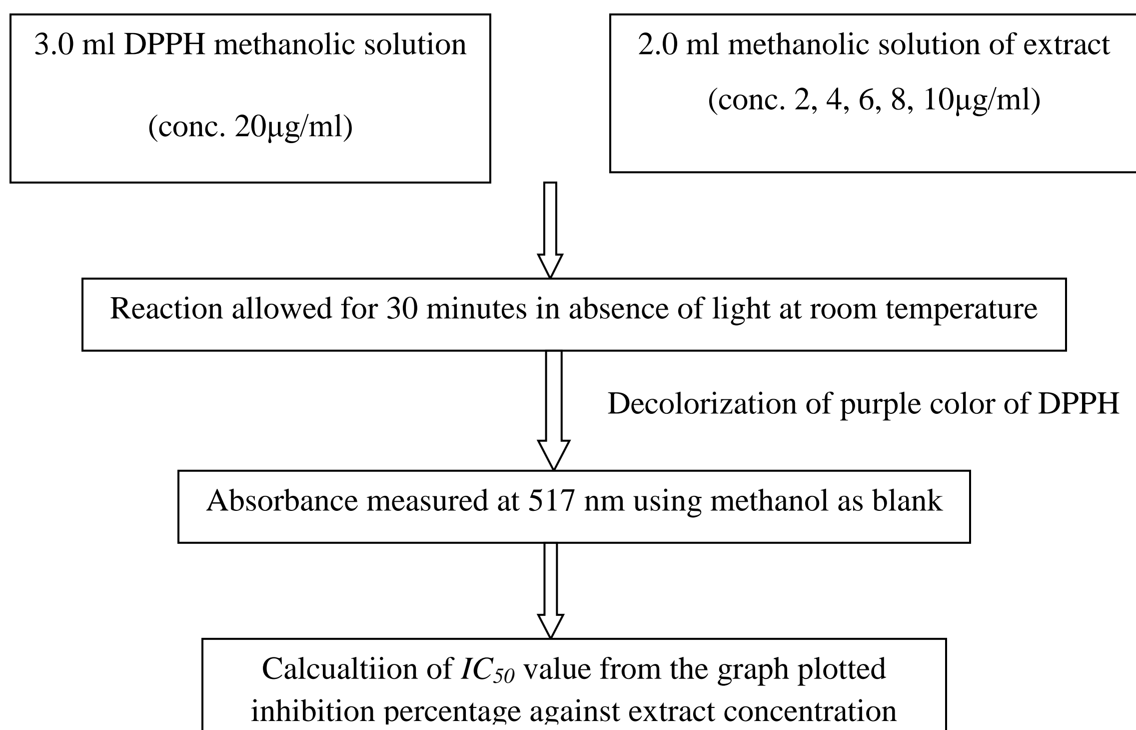


Figure 6. 1 : Schematic representation of the method of assaying free radical scavenging activity

6.1.4. Results: Table 6.1: Results of percent inhibition by the extracts.

Name of Extract	Concentrations	Absorbance	% Inhibition	IC $_{50}$
SPSA 1	2 μg/ml	0.067	44.63	2.10 μg/ml
SPSA 2	4 μg/ml	0.032	73.55	
SPSA 3	6 μg/ml	0.017	85.95	
SPSA 4	8 μg/ml	0.013	89.26	
SPSA 5	10 μg/ml	0.010	91.74	
SPSN 1	2 μg/ml	0.062	48.76	3.40 μg/ml
SPSN 2	4 μg/ml	0.048	60.33	
SPSN 3	6 μg/ml	0.037	69.42	
SPSN 4	8 μg/ml	0.029	76.03	
SPSN 5	1 0μg/ml	0.021	82.64	
SPSD 1	2 μg/ml	0.063	47.93	1.72 μg/ml
SPSD 2	4 μg/ml	0.024	80.17	
SPSD 3	6 μg/ml	0.019	84.30	
SPSD 4	8 μg/ml	0.015	87.60	
SPSD 5	10 μg/ml	0.013	89.27	
SPSE 1	2 μg/ml	0.106	12.40	5.47 μg/ml
SPSE 2	4 μg/ml	0.091	24.79	
SPSE 3	6 μg/ml	0.068	43.80	
SPSE 4	8 μg/ml	0.026	78.51	
SPSE 5	10 μg/ml	0.025	79.34	

Absorbance of control, $A_{control} = 0.121$

N.B:

SPSN = *Spondias pinnata* (n-Hexane)

SPSE = *Spondias pinnata* (Ethyl Acetate)

SPSD = *Spondias pinnata* (DCM)

SPSA = *Spondias pinnata* (Aqueous)

6.1.5. Discussion

Different partitionates of ethanolic extract of *S. pinnata* fruit skin were subjected to free radical scavenging activity.

In this investigation, the dichloromethane soluble partitionate of the *S. pinnata* skin showed the highest free radical scavenging activity with IC_{50} value 1.72 µg/ml.

Table 6.2: Determination of free radical scavenging capacity for dichloromethane fraction

Name of Extract	Concentrations	Absorbance	% Inhibition	IC_{50}
SPSD 1	2 µg/ml	0.063	47.93	1.72µg/ml
SPSD 2	4 µg/ml	0.024	80.17	
SPSD 3	6 µg/ml	0.019	84.30	
SPSD 4	8 µg/ml	0.015	87.60	
SPSD 5	10 µg/ml	0.013	89.27	

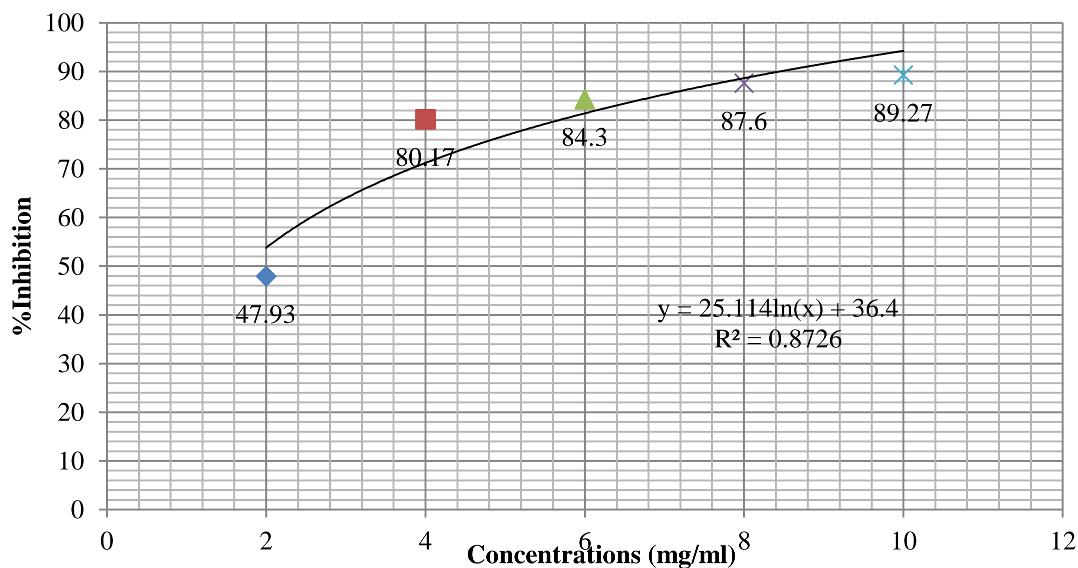


Figure 6. 2: IC_{50} value calculation for *S. pinnata* dichloromethane fraction

Aqueous fraction of the ethanolic extract also revealed potent scavenging activity IC_{50} 2.10 µg/ml.

Table 6.3: Determination of free radical scavenging capacity for aqueous fraction

Name of Extract	Concentrations	Absorbance	% Inhibition	IC_{50}
SPSA 1	2 µg/ml	0.067	44.63	2.10 µg/ml
SPSA 2	4 µg/ml	0.032	73.55	
SPSA 3	6 µg/ml	0.017	85.95	
SPSA 4	8 µg/ml	0.013	89.26	
SPSA 5	10 µg/ml	0.010	91.74	

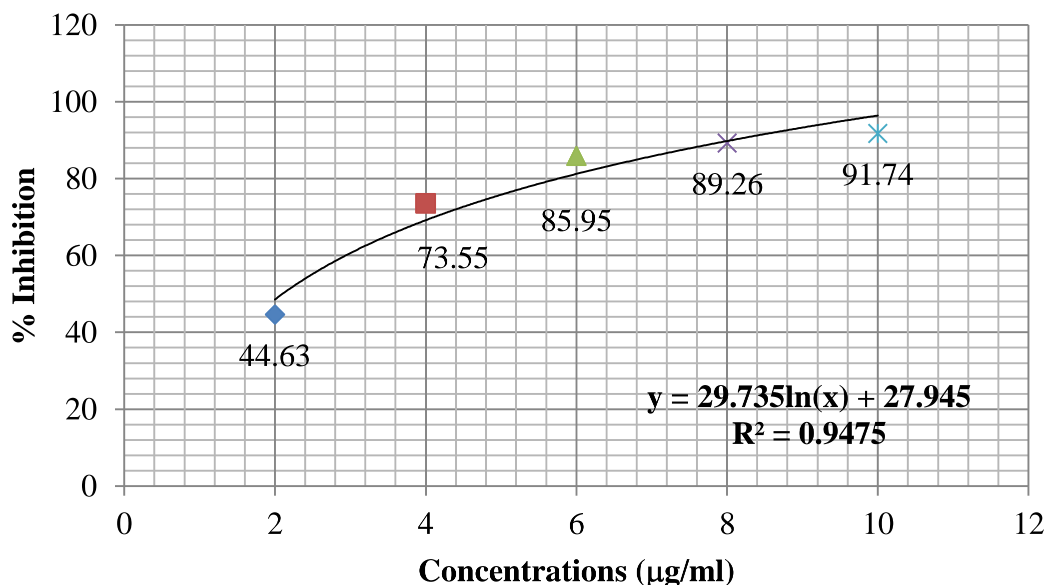


Figure 6. 3: IC_{50} value calculation for *S. pinnata* fruit skin of aqueous fraction

At the same time the n-hexane soluble partitionate also exhibited strong antioxidant potential having IC_{50} value 3.41 µg/ml aqueous and ethyl acetate soluble fraction of the ethanolic extract also revealed potent scavenging activity IC_{50} 3.41 µg/ml and 6 µg/ml respectively.

Table 6.4: Determination of free radical scavenging capacity for n-hexane fraction

Name of Extract	Concentrations	Absorbance	% Inhibition	IC_{50}
SPSN 1	2 μg/ml	0.085	29.75	3.41μg/ml
SPSN 2	4 μg/ml	0.048	60.33	
SPSN 3	6 μg/ml	0.037	69.42	
SPSN 4	8 μg/ml	0.029	76.03	
SPSN 5	10 μg/ml	0.021	82.64	

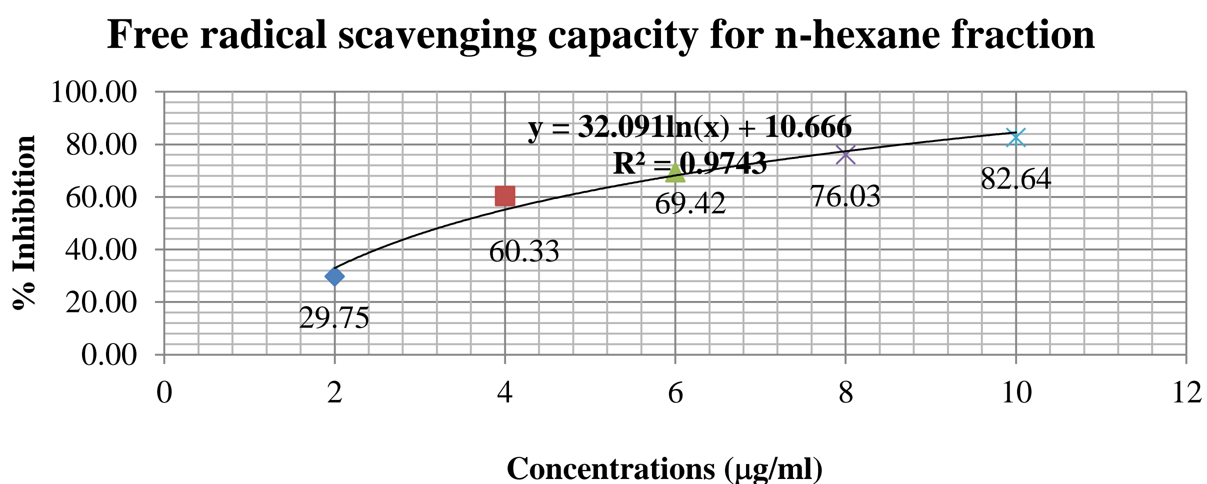


Figure 6. 4: IC_{50} value calculation for *S. pinnata* n-hexane fraction

Ethyl acetate soluble fraction of the ethanolic extract also revealed potent scavenging activity 5.47 μg/ml.

Table 6.5: Determination of free radical scavenging capacity for Ethyl acetate fraction

Name of Extract	Concentrations	Absorbance	% Inhibition	IC_{50}
SPSE 1	2 μg/ml	0.106	12.40	5.47 μg/ml
SPSE 2	4 μg/ml	0.091	24.79	
SPSE 3	6 μg/ml	0.068	43.80	
SPSE 4	8 μg/ml	0.026	78.51	
SPSE 5	10 μg/ml	0.025	79.34	

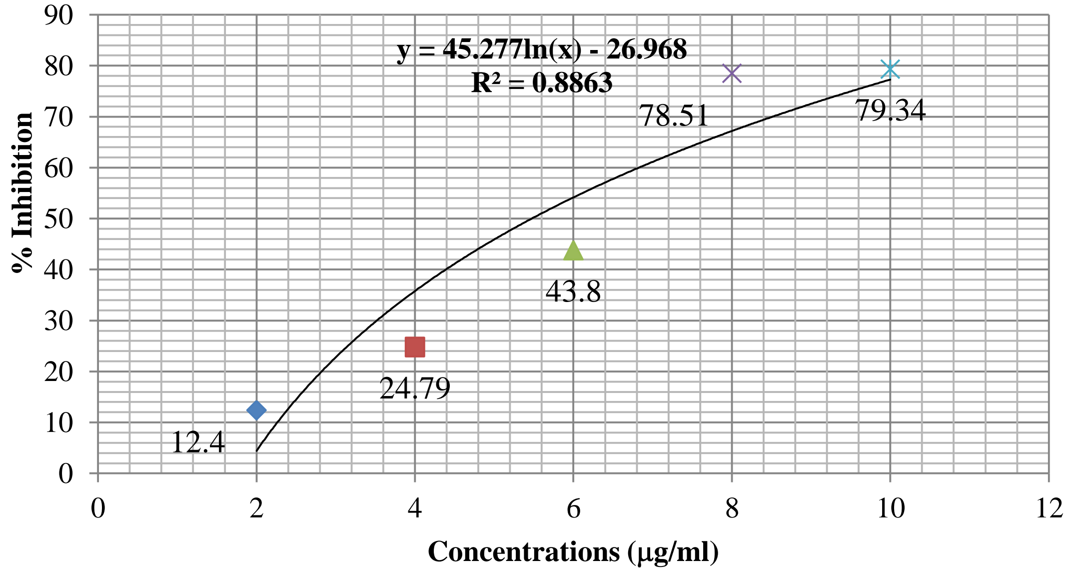


Figure 6. 5: IC_{50} value calculation for *S. pinnata* Ethyl acetate fraction

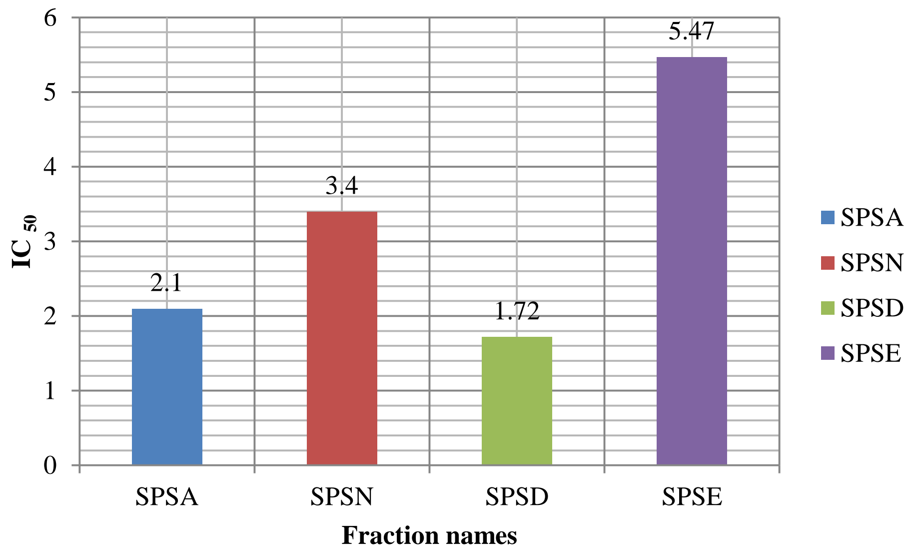


Figure 6. 6 : Comparison of IC_{50} value of different extracts

6.2. Determination of total phenolic content

6.2.1. Principle

The total phenolic concentration of the extract of *Spondias pinnata* skin was determined by the modified Folin-Ciocalteu method (Wolfe *et al.,* 2003). The process of measuring total phenolic content of the crude extract of *Spondias pinnata* fruit involves the use of Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It measures the amount of substance being tested needed to inhibit the oxidation of the Folin-Ciocalteu reagent. The reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically. The generated chromogens give a strong absorption maximum at 760 nm.

6.2.2.2. Materials & Reagents

Test tube
Pipette
Spatula
Folin-Ciocalteu reagent
Sodium carbonate (Na_2CO_3)
Gallic acid
Analytical balance
UV- visible spectrophotometer
Vortex mixer
Distilled water
Methanol
Aluminium foil

6.2.2.3. Methods

- 0.5ml of a methanol solution of the crude extract of concentration of 1mg/ml was mixed with 5ml Folin ciocalteu reagent (1:10 v/v distilled water) and 4 ml (75g/L) of Sodium carbonate.
- The mixture was vortexed for 15 second and allowed to stand for 30min at room temperature in dark place for color development and the absorbance was measured at 760 nm against methanol as blank by using a UV- visible spetrophotometer.
- The total phenolics was expressed as gm of GAE (gallic acid equivalent)/100gm of the dried extract using the following equation obtained from a standard Gallic acid calibration curve:

  $y = 0.0162x+0.0215$, $R^2=0.9972$.

6.2.3. Result :Table 6.6: Results of total phenolic content

Sample name	Conc.(mg/ml)	Absorbance	Total Phenolic content(mg of GAE/gm of dried extract)	Average
Amra skin(n-Hexane)1	1	0.359	393.082	409.387375
Amra skin(n-Hexane)2	1	0.387	425.693	
Amra skin(DCM)1	1	0.432	478.104	513.626834
Amra skin(DCM)2	1	0.493	549.15	
Amra skin(aq.)1	1	0.475	528.185	570.696483
Amra skin(aq.)1	1	0.548	613.208	
Amra skin(EtAc)	1	0.3	324.365	336.012113
Amra skin(EtAc)	1	0.32	347.659	

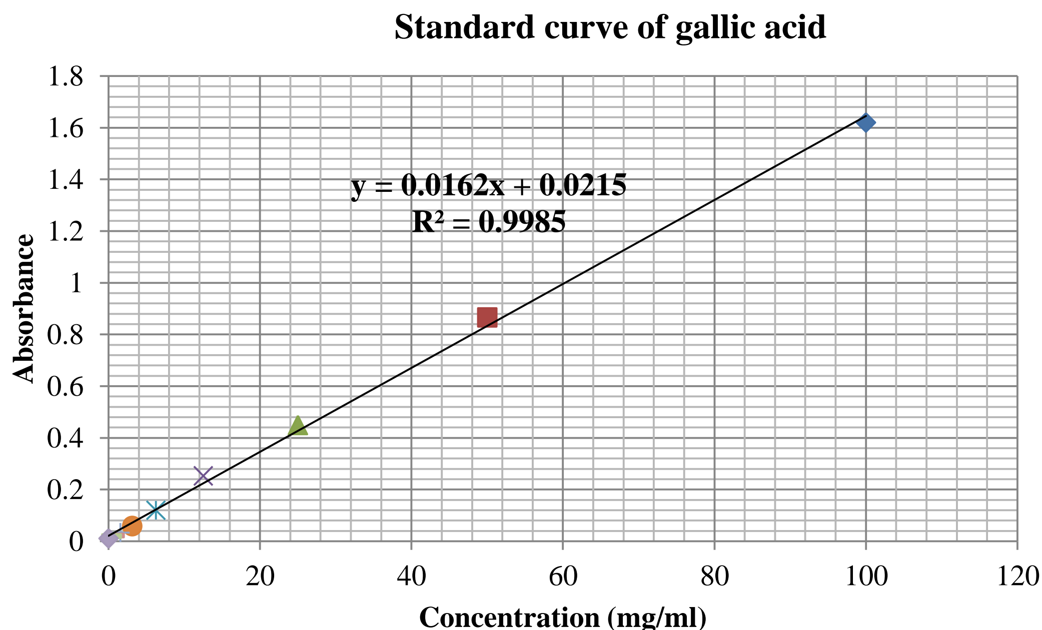


Figure 6. 7 : Standard curve of gallic acid

6.2.4. Discussion

Aqueous fraction of the ethanolic extract contains highest amount of phenolic compound. It contains average of 570.696 mg equivalent of GAE/gm.

Dichloromethane fraction of the ethanolic extract also contains good amount of phenolic compound. It contains average of 513.6267 mg equivalent of GAE/gm.

n-hexane fraction of the ethanolic extract contains average of 409.387mg equivalent of GAE/gm.

Ethyl acetate fraction have 336.012113 mg equivalent of GAE/gm.

Ethyl acetate fraction of the ethanolic extract contains average of 345.115mg equivalent of GAE/gm.

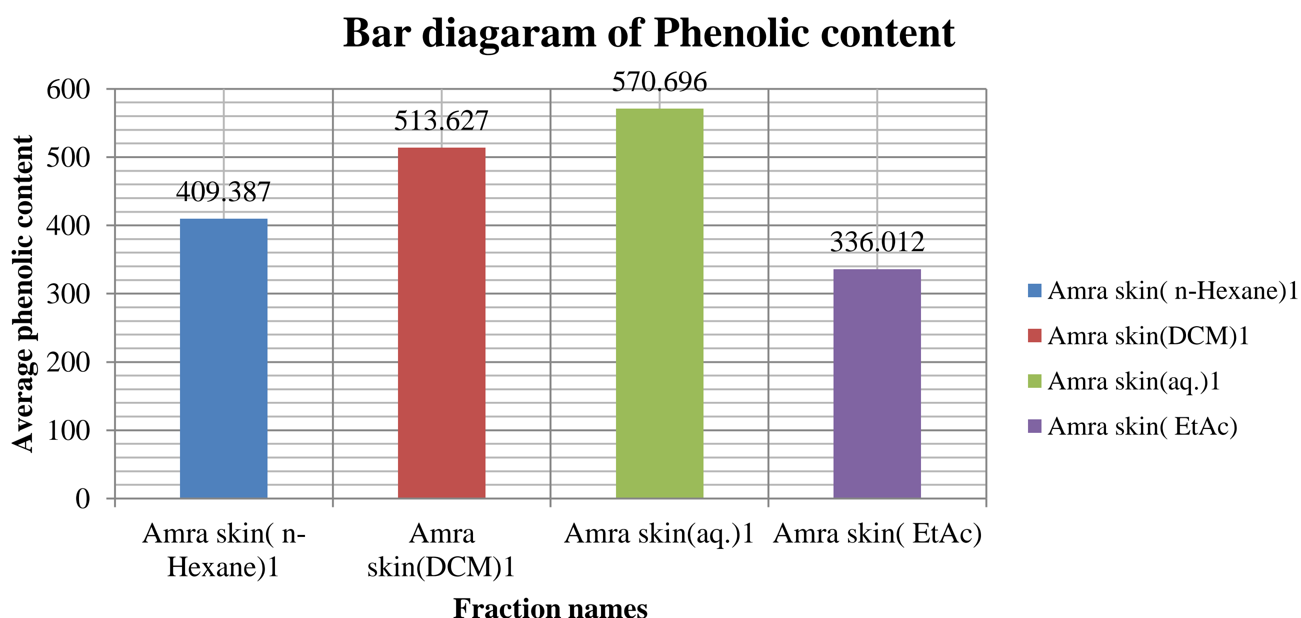


Figure 6. 8: Bar diagram of phenolic content in different fractions of *Spondias pinnata*

6.3. Determination of reducing power assay

6.3.1. Principle

The reducing power assay of the extract of *Spondias pinnata* fruit was determined according to the method previously described by (Oyaizu *et.al.,* 1986). Reducing power assay is based on the

principle that substances which have reduction potential react with potassium ferricyanide [**$K_3Fe(CN)_6$**] to form potassium ferrocyanide [**$K_4Fe(CN)_6$**], which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard.

6.3.2. Materials & Reagents

Test tube	Analytical balance
Pipette	UV- visible spectrophotometer
Spatula	Hot air oven
Vortex mixer	Centrifuge machine
0.2 M Phosphate buffer	Potassium ferricyanide [**$K_3Fe(CN)_6$**]
10 % Trichloroacetic acid (TCA)	Distilled water
Ferric chloride **($FeCl_3$)**	L-ascorbic acid

6.3.3. Methods

- 1 ml of the methanol solution of the crude extract of different concentrations (1, 5, 10, 50, 100µg/mL) was mixed with 2.5 ml phosphate buffer and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%).
- The mixture was incubated at 50°C for 20min.
- 2.5mL of trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000rpm for 10min.
- The upper layer of the solution was separated and mixed with 2.5 ml distilled water and 0.5ml $FeCl_3$.
- The absorbance was measured against a blank at 700nm.
- All the tests were carried out in triplicate and average absorption was noted for each time.

- L-Ascorbic acid was used as positive control.
- Percentage (%) increase in reducing power was calculated as follows:

  % Increase in reducing power = $(A_{Test} / A_{Blank}) - 1 \times 100$

  Where A_{Blank} is absorbance of blank (containing all reagents except the test material)

  A_{Test} is absorbance of test solution.

6.3.4. Result

Table 6.7: Result of reducing power test

Sample	Concentration µg/ml	Absorbance	Absorbance of blank, A_{Blank}	% Reducing power = $(A_{Test} / A_{Blank}) - 1 \times 100$
Amra Skin (n-Hexane)	1	0.093	0.089	4.49
	5	0.158		77.53
	10	0.257		188.76
	50	0.565		534.83
	100	0.735		725.84
Amra Skin (DCM)	1	0.097		8.99
	5	0.102		14.61
	10	0.260		192.13
	50	0.534		500.00
	100	0.835		838.20
Amra Skin (Ethyl Acetate)	1	0.092		3.37
	5	0.107		20.22
	10	0.357		301.12
	50	0.589		561.80
	100	0.632		610.11
Amra Skin (Aq)	1	0.099		11.24
	5	0.123		38.20
	10	0.153		71.91
	50	0.196		120.22
	100	0.372		317.98

6.3.5. Discussion

- Here, the entire fraction produces good reducing power capability. Higher absorbance indicates formation of high amount of ferrous complex due to reduction of potassium ferricyanide into potassium ferrocyanide. Percentage (%) increase in reducing power was calculated as follows: **% Increase in reducing power = (A_{Test} / A_{Blank}) -1 X 100**

Table 6.8: Reducing power of dichloromethane fraction

Sample	Concentration µg/ml	Absorbance	Absorbance of blank, A_{Blank}	% Reducing power = (A_{Test} / A_{Blank}) -1 X 100
Amra Skin (Dichloromethane)	1	0.097	0.089	8.99
	5	0.102		14.61
	10	0.260		192.13
	50	0.534		500.00
	100	0.835		838.20

Dichloromethane fraction produces highest reduction at 100 µg/ml concentrations and produces absorbance of 8.99, 14.61, 192.13, and 500 for 1, 5, 10, and 50 µg/ml concentrations respectively.

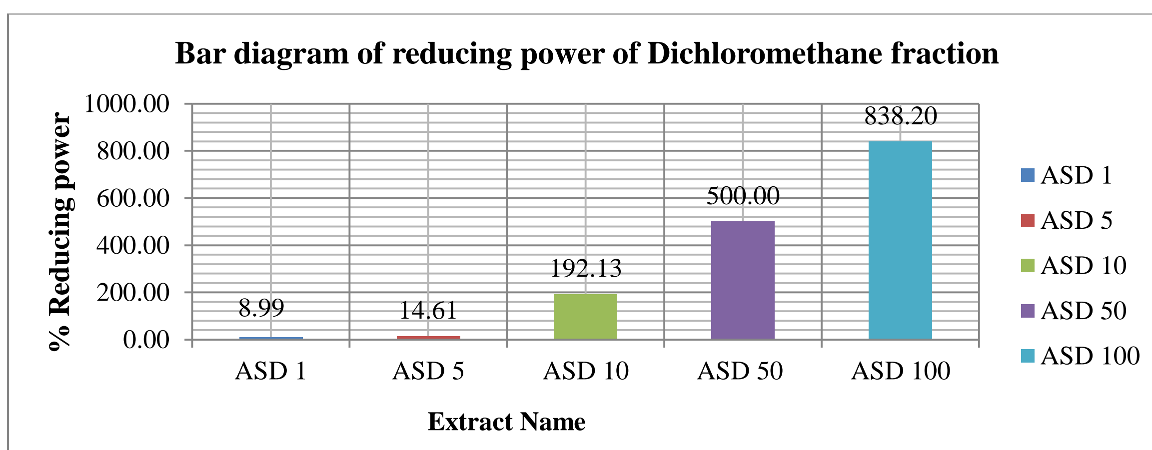


Figure 6. 9: Bar diagram showing percent reducing power of Dichloromethane fraction (ASD= Amra Skin Dichloromethane Fraction)

Table 6.9: Reducing power of aqueous fraction

Sample	Concentration µg/ml	Absorbance	Absorbance of blank, A_{Blank}	% Reducing power = (A_{Test} / A_{Blank}) -1 X 100
Amra Skin (Aqueous)	1	0.099	0.089	11.24
	5	0.123		38.20
	10	0.153		71.91
	50	0.196		120.22
	100	0.372		317.98

Aqueous fraction has highest reducing capability of 11.24 among all 1 µg/ml concentrations and having percent reducing power of 38.20, 71.91, 120.22 and 317.98 for 5, 10, 50 and 100µg/ml concentration respectively.

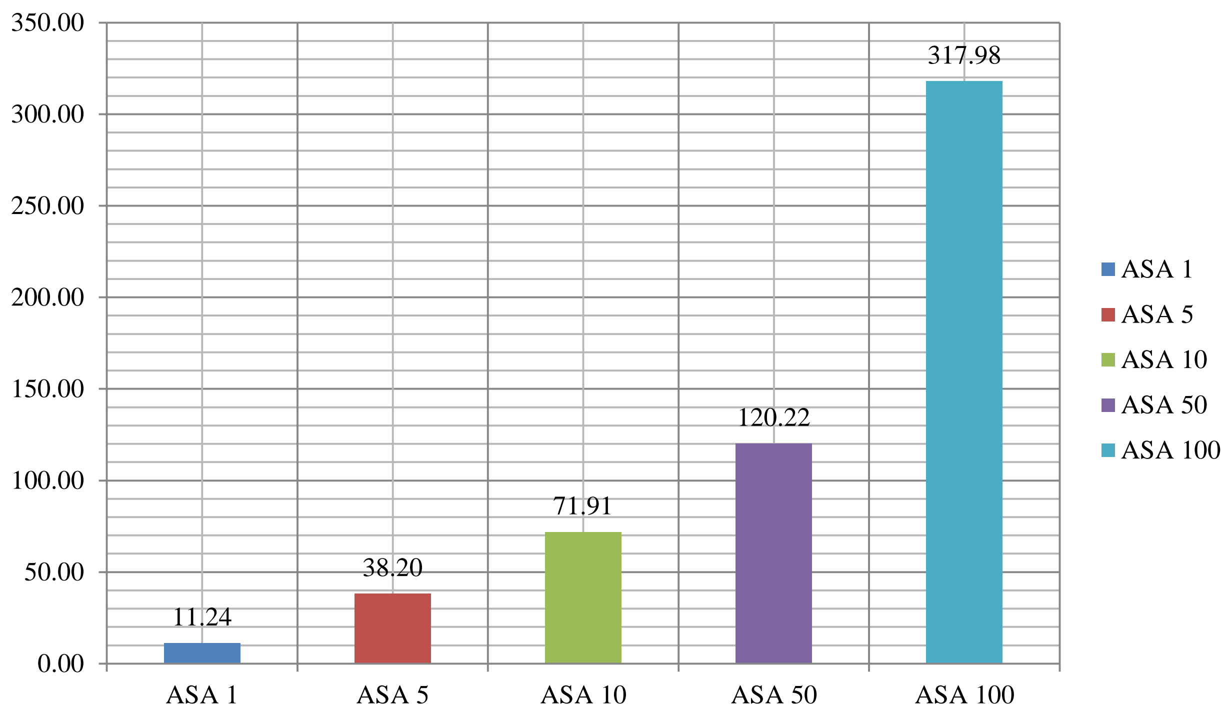


Figure 6. 10: Bar diagram showing percent reducing power of Aqueous fraction (ASA= Amra Skin Aqueous Fraction)

Table 6.10: Reducing power of ethyl acetate fraction

Sample	Concentration µg/ml	Absorbance	Absorbance of blank, A_{Blank}	% Reducing power = (A_{Test} / A_{Blank}) -1 X 100
Amra Skin (Ethyl Acetate)	1	0.092	0.089	3.37
	5	0.107		20.22
	10	0.357		301.12
	50	0.589		561.80
	100	0.632		610.11

Ethyl acetate fraction has highest percentage of reducing power (301.12) among all 10 µg/ml concentrations of the fractions and produces percent reducing power of 3.37, 20.22, 561.80 and 610.11 for 1, 5, 50 and 100µg/ml concentration respectively.

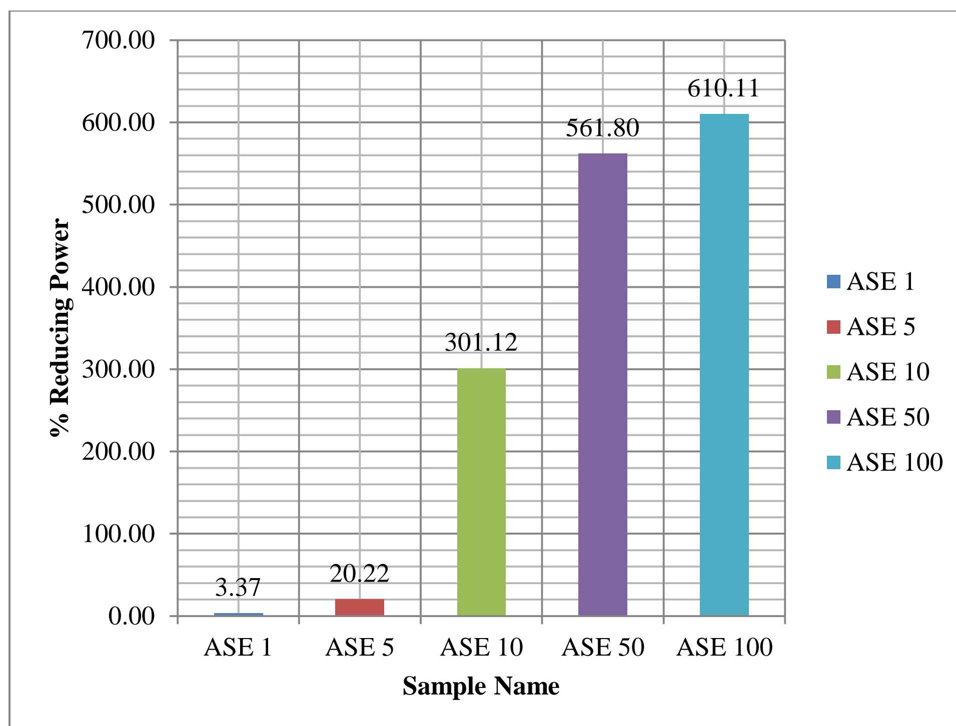


Figure 6. 11 : Bar diagram showing percent reducing power of Ethyl acetate (ASE= Amra Skin Ethyl Acetate Fraction)

Table 6.11: Reducing power of n-hexane fraction

Sample	Concentration µg/ml	Absorbance	Absorbance of blank, A_{Blank}	% Reducing power = (A_{Test} / A_{Blank}) -1 X 100
Amra Skin (n-Hexane)	1	0.093	0.089	4.49
	5	0.158		77.53
	10	0.257		188.76
	50	0.565		534.83
	100	0.735		725.84

n-hexane fraction shows highest reducing power (77.53) at 5 µg/ml concentration and produces percent reduction of 4.49, 188.76, 534.83 and 725.84 for 1, 10, 50 and 100µg/ml concentration respectively.

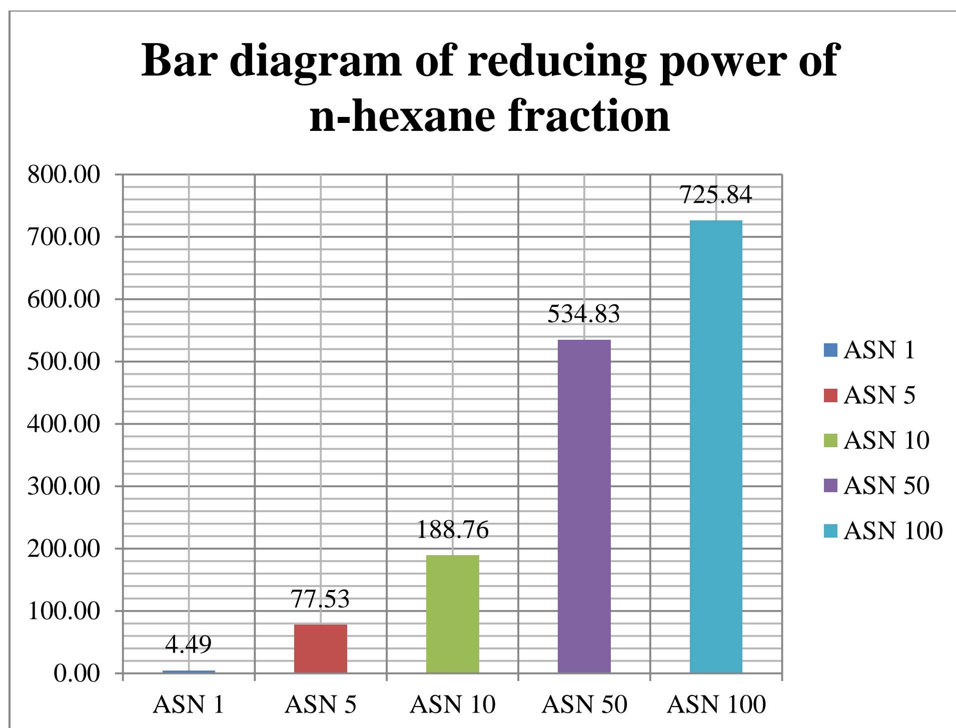


Figure 6. 12: Bar diagram showing percent reducing power of n-hexane fraction (ASN= Amra Skin n-hexane Fraction)

6.4. Evaluation of total antioxidant activity by phosphomolybdenum method

The total antioxidant capacity of crude ethanolic plant extract was determined by phosphomolybdenum method. Sometimes a correlation analysis is performed between the total phenolic content and total antioxidant capacity to reveal the correlation. It is obvious that the plant phenolic compounds contribute to the the major antioxidant activity. Based on the absorbance values of the extract solution, reacted with reagent solution (0.6 M Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate) and compared with the standard solutions of L-ascorbic acid equivalents, result of the colorimetric analysis of the total antioxidant capacity is given in table 6.9. Total antioxidant capacity of the sample is expressed as mg of L-ascorbic acid per gm of dried extract. The total antioxidant capacity found in the crude ethanolic extract of *Spondias pinnata* (skin) was 25.52 gm of L-ascorbic acid per gm of dried extract.

Table 6.12: Standard curve preparation by using L-ascorbic acid

Serial no.	Concentrations (mg/ ml)	Absorbance	Regression line	R^2
1	2	0.12	y = 0.113x - 0.146	0.9916
2	4	0.27		
3	6	0.5		
4	8	0.77		
5	10	1.0		

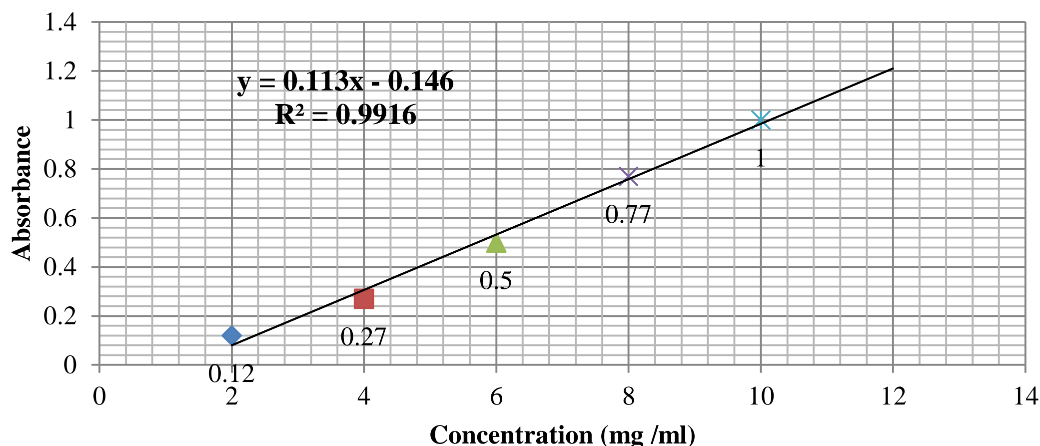


Figure 6.13: Standard curve for ascorbic acid

6.4.1. Result

Table 6.13: Results of phosphomolybdenum test

Sample name	Sample number	Concentrations (mg/ml)	Absorbance	gm of L-ascorbic acid equivalent per g of dried extract	Average
ASD	1	2	0.163	15.0248	
ASD	2	2	0.178	15.75416	
ASD	3	2	0.160	14.87893	15.21929
ASN	1	2	0.278	20.61655	
ASN	2	2	0.284	20.9083	
ASN	3	2	0.333	23.29087	21.60524
ASE	1	2	026	19.74132	
ASE	2	2	026	19.74132	
ASE	3	2	0.27	20.22756	19.9034
ASA	1	2	0.56	9.822036	
ASA	2	2	0.54	9.724788	
ASA	3	2	0.53	9.676165	9.740996

Absorbance of control, $A_{control}$ = 0.121 ASN = *Spondias pinnata* (n-Hexane)ASE = *Spondias pinnata* (Ethyl Acetate)ASD = *Spondias pinnata* (DCM) ASA = *Spondias pinnata* (Aqueous)

6.4.2. Discussion

The n-hexane fraction produces the highest antioxidant activity with an average of 21.61 gm of L-ascorbic acid equivalent per g of dried extract. The ethyl acetate, dichloromethane and aqueous fraction also produce good activity of 19.9034, 15.21929 and 9.740996 gm of L-ascorbic acid equivalent per g of dried extract respectively.

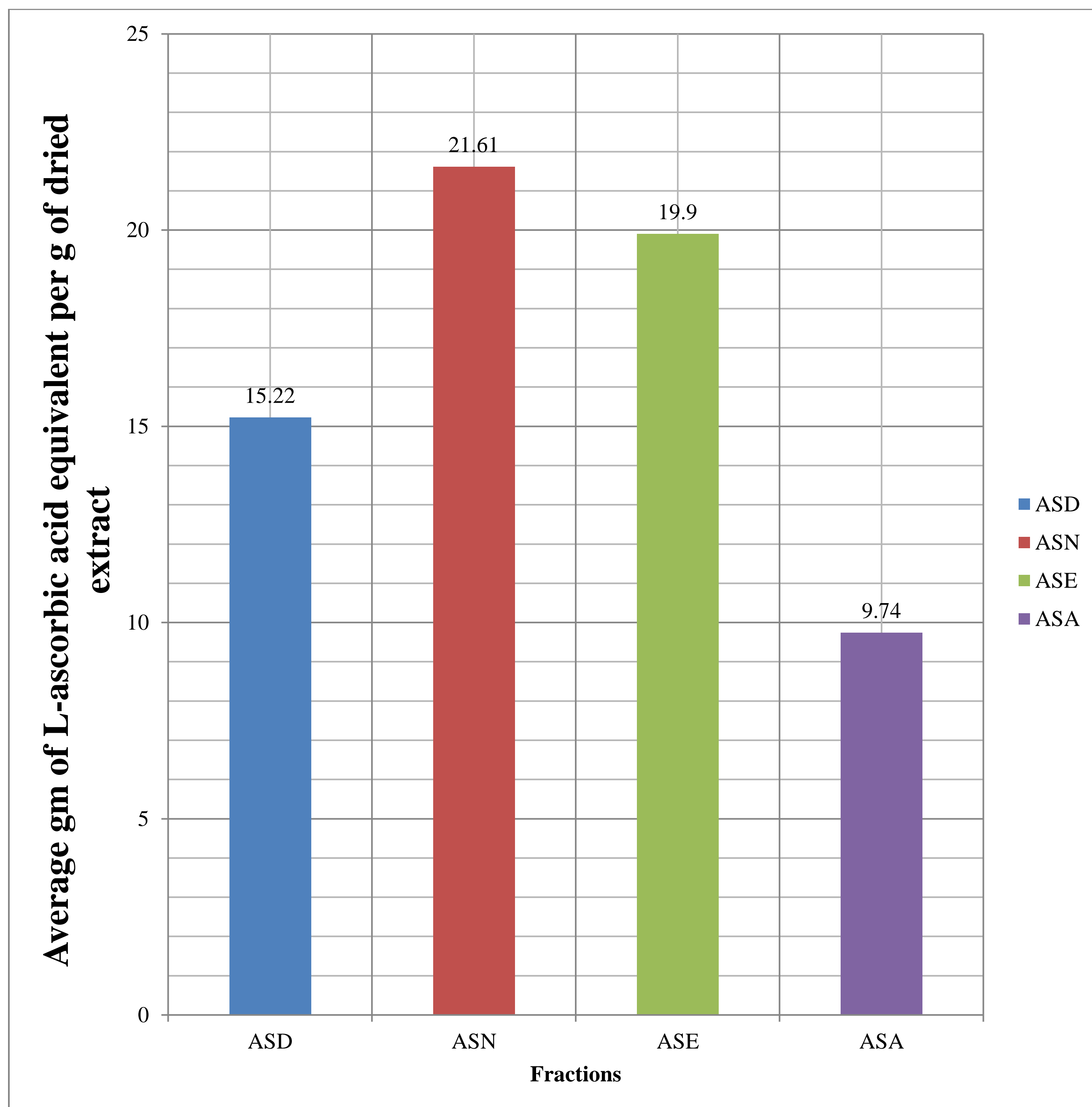


Figure 6.14: Bar Diagram of phosphomolybdenum test

6.5. Assay for total flavonoids concentration (Chang C, 2002)

Aluminium chloride colorimetric method (Chang *et al.,* 2002) was used for determination of total flavonoids concentration in the samples of *S. pinnata* fruit skin. Each extract and fraction (0.5 ml, 1:10 gml^{-1}) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water It was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm. Total flavonoids content was determined as mg of Quercetin equivalent per gram using the equation obtained from a standard Quercetin calibration curve $y=0.002x + 0.0318$; $R^2 = 0.9989$

6.5.1. Materials and Equipment's

- Test tubes
- UV-2450 spectrophotometer
- Test tube holder.
- Beaker
- Electronic balance

6.5.2. Reagents

- Methanol
- Aluminum chloride
- Potassium acetate
- Quercetin
- Distilled water

6.5.3. Results

Table 6.14: Results of total flavonoid content

Name of the fraction	Absorbance	Total flavonoid content(mg of Quercetin/gm of dried extract)	Average (mg of Quercetin/gm of dried extract)
ASN 1	0.278	123.1	133.2667
ASN 2	0.284	126.1	
ASN 3	0.333	150.6	
ASA 1	0.056	12.1	11.26667
ASA 2	0.054	11.1	
ASA 3	0.053	10.6	
ASD 1	0.16	64.1	67.6
ASD 2	0.178	73.1	
ASD 3	0.163	65.6	
ASE 1	0.26	114.1	115.7667
ASE 2	0.26	114.1	
ASE 3	0.27	119.1	

Absorbance of control, $A_{control} = 0.121$ ASN = *Spondias pinnata* (n-Hexane)ASE = *Spondias pinnata* (Ethyl Acetate)ASD = *Spondias pinnata* (DCM) ASA = *Spondias pinnata* (Aqueous)

6.5.4. Discussion

Table 6.15: Standard curve preparation by using quercetin

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	2.5	0.0365	y = 0.002x + 0.0318	0.9989
2	5	0.0417		
3	10	0.0521		
4	20	0.0735		
5	30	0.0906		
6	40	0.1127		

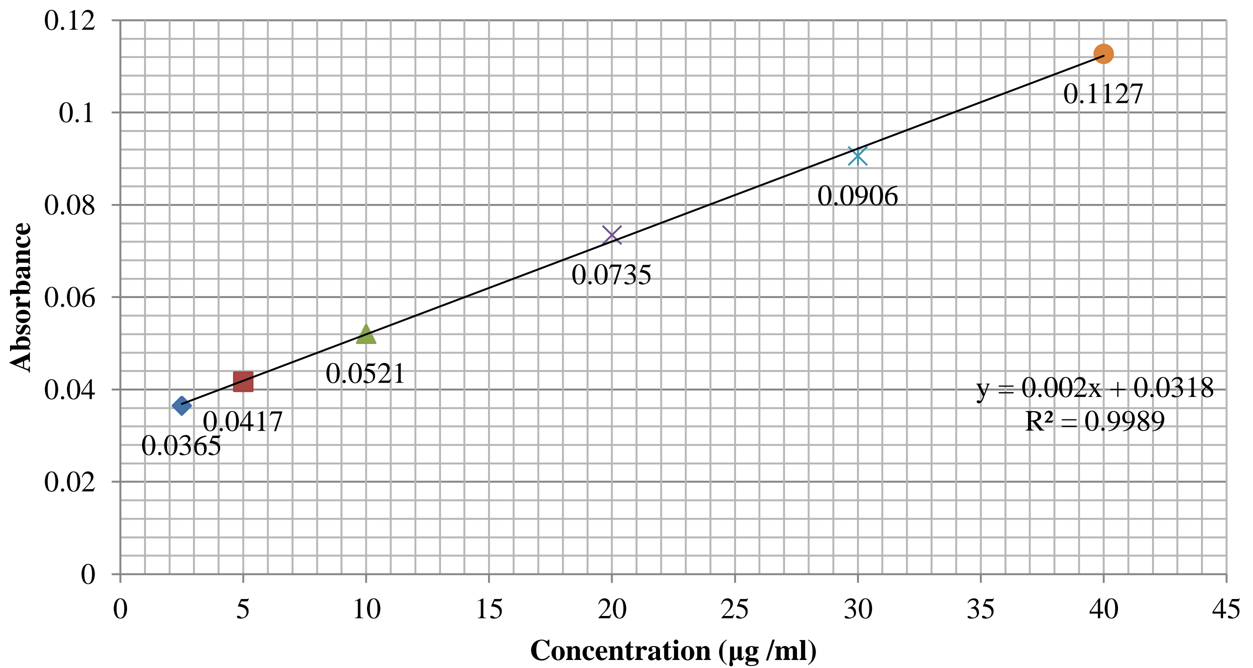


Figure 6.15: Standard curve for Quercetin

So, from the result obtained we can conclude that, n- hexane soluble fraction contains highest amount of flavonoid having the average of 133.267 mg of Quercetin/gm of dried extract. The ethyl acetate fraction of the dry extract contains 115.7667 mg of Quercetin/gm of dried extract.

But, the dichloromethane and aqueous fraction contains 67.6 mg of Quercetin/gm of dried extract and 11.2667 mg of Quercetin/gm of dried extract respectively.

It indicates that, most of the flavonoids present in the ethanolic extracts of the *S. pinnata* are lipophilic in nature.

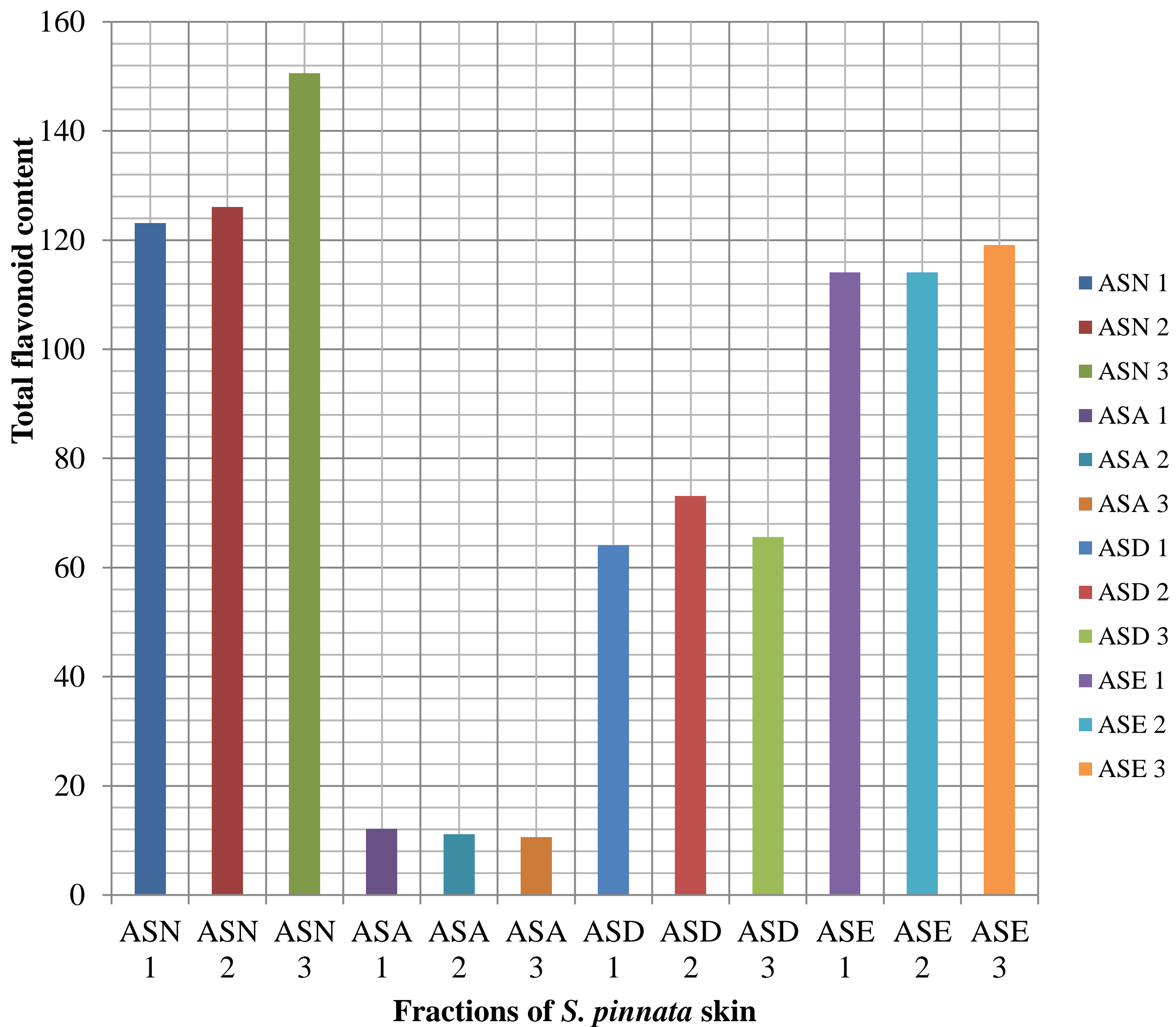


Figure 6.16: Comparison of the flavonoid content in different fractions of *S. pinnata* skin

Chapter-7: Study of Thrombolytic activity

7.1. Thrombolytic activity study (Prasad *et* al, 2006)

Thrombolytic therapy reduces mortality and preserves left ventricular function in patients with myocardial infarction. Streptokinase is widely used fibrinolytic drug that was used in this study as standard. All thrombolytic agents work by activating the enzyme plasminogen that clears the cross-linked fibrin mesh.

In our study ethanolic extract was used as experimental drug. 5ml of blood samples were collected from volunteer and distributed into three separate pre-weighed (W_1) epppendrof tubes. The blood specimens were incubated for 45 minutes at 37°C. After clotting of blood, serum was decanted and removed. Then weight of clotted blood (ΔW) was taken by subtracting the pre-weight (W_1) from the weight of clot containing tube (W_2) as - $\Delta W = W_2 – W_1$.

Then 100µl extract solution of *S. pinnata* skin was added to the clot containing tube. Similarly 100µl of streptokinase was added to clot of standard tube and 100µl of water was added to clot of blank tube those were used as positive and negative control respectively. Then all the tubes were incubated at 37°C for 90 minutes and weighed again for getting the weight variation among the pre weight and final weight (W_3) that was achieved for clot lyses (thrombolysis).

Weight loss of clot after application of extract solution was taken as the functional indication of thrombolytic activity. The study was implemented on two volunteer with five blood samples (for each) of mid-age. Average value of weight loss (in %) was calculated to examine the variation of two volunteer. Percentage of clot lysis was calculated with the following formula –

% of clot lysis= weight of released clot/clot weight . 100%

7.2. Materials

1. Blood from volunteer
2. Electronic balance
3. Syringe
4. Eppendrof tube
5. Beaker
6. Streptrokinase

7.3. Results

Table 7.1: Results of the thrombolytic activity

Name of Extract	Wt of empty Eppendrof (W_1) gm	Wt of Eppendrof with clot (W_2) gm	Wt of clot (W_2-W_1) gm	Wt of Eppendrof after clot lysis (W_3) gm	Wt of released clot (W_2-W_3) gm	% of Clot lysis
BlankV1	0.881	1.167	0.286	1.162	0.005	1.7482
BlankV2	0.886	1.115	0.229	1.111	0.004	1.7467
StreptokinaseV1	0.886	1.122	0.236	0.998	0.124	52.5424
StreptokinaseV1	0.876	1.162	0.286	0.999	0.163	56.993
StreptokinaseV1	0.892	1.129	0.237	0.995	0.134	56.5401
StreptokinaseV2	0.865	1.118	0.253	0.992	0.126	49.8024
StreptokinaseV2	0.895	1.117	0.222	1.001	0.116	52.2523
StreptokinaseV2	0.882	1.123	0.241	1.002	0.121	50.2075
SPAV1	0.878	0.957	0.079	0.949	0.008	10.1266
SPAV1	0.893	0.961	0.068	0.954	0.007	10.2941
SPAV1	0.881	0.959	0.078	0.951	0.008	10.2564
SPAV2	0.885	0.952	0.067	0.945	0.007	10.4478
SPAV2	0.953	1.141	0.188	1.122	0.019	10.1064
SPAV2	0.892	1.1	0.208	1.079	0.021	10.0962
SPEV1	0.885	1.12	0.235	1.095	0.025	10.6383
SPEV1	0.901	1.14	0.239	1.114	0.026	10.8787
SPEV1	0.899	1.103	0.204	1.081	0.022	10.7843
SPEV2	0.883	1.042	0.159	1.025	0.017	10.6918
SPEV2	0.903	1.136	0.233	1.111	0.025	10.7296
SPEV2	0.936	1.324	0.388	1.283	0.041	10.5670
SPDV1	0.871	1.999	1.128	1.71	0.289	25.6206
SPDV1	0.873	1.867	0.994	1.61	0.257	25.8551
SPDV1	0.872	1.954	1.082	1.68	0.274	25.3235
SPDV2	0.871	1.116	0.245	1.053	0.063	25.7143
SPDV2	0.887	1.064	0.177	1.018	0.046	25.9887
SPDV2	0.882	1.119	0.237	1.06	0.059	24.8945
SPNV1	0.872	1.162	0.290	1.084	0.078	26.8966
SPNV1	0.876	1.334	0.458	1.207	0.127	27.7293
SPNV1	0.863	1.181	0.318	1.096	0.085	26.7296
SPNV2	0.873	1.222	0.349	1.112	0.11	31.5186
SPNV2	0.864	1.134	0.27	1.033	0.101	37.4074
SPNV2	0.880	1.097	0.217	1.024	0.073	33.6406

N.B SPAV= *Spondias pinnata* Aqueous fraction volunteer, SPDV= *Spondias pinnata* Dichloromethane fraction volunteer, SPEV= *Spondias pinnata* Ethyl acetate fraction volunteer, SPNV= *Spondias pinnata* n-hexane fraction volunteer

7.4. Discussion

Streptokinase is used as standard. The average clot lysis by streptokinase is 55.35849 in volunteer 1 and 50.75403 for volunteer 2.

The n-hexane fraction of the ethanolic extract produces best activity among other extracts. The average clot lysis activity of the fraction is found to be 27.11846 for volunteer 1 and 34.18886 for volunteer 2.

Table 7.2: Clot lysis by n-hexane fraction of the *S. pinnata* skin

Name of Extract	**Wt of empty Eppendrof (W_1) gm**	**Wt of Eppendrof with clot (W_2) gm**	**Wt of clot (W_2-W_1) gm**	**Wt of Eppendrof after clot lysis (W_3) gm**	**Wt of released clot (W_2-W_3) gm**	**% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)**	**Average**
SPNV1	0.872	1.162	0.290	1.084	0.078	26.8966	27.11846
SPNV1	0.876	1.334	0.458	1.207	0.127	27.7293	
SPNV1	0.863	1.181	0.318	1.096	0.085	26.7296	
SPNV2	0.873	1.222	0.349	1.112	0.11	31.5186	34.18886
SPNV2	0.864	1.134	0.27	1.033	0.101	37.4074	
SPNV2	0.880	1.097	0.217	1.024	0.073	33.6406	

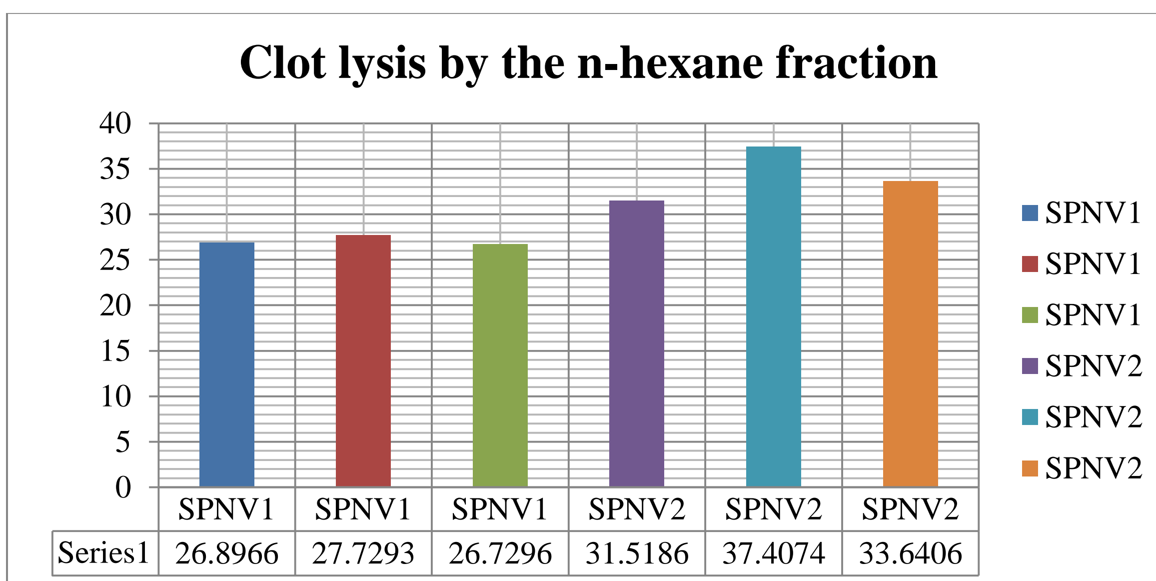


Figure 7.1: Bar diagram of the percent clot lysis by the n-hexane fraction

Dichloromethane partitionate also produces good result having average values of clot lysis are 25.59972 for volunteer 1 and 25.5325 for volunteer 2.

Table 7.3: Clot lysis by dichloromethane fraction of the *S. pinnata* skin

Name of Extract	**Wt of empty Eppendrof (W_1) gm**	**Wt of Eppendrof with clot (W_2) gm**	**Wt of clot (W_2-W_1) gm**	**Wt of Eppendrof after clot lysis (W_3) gm**	**Wt of released clot (W_2-W_3) gm**	**% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)**	**Average**
SPDV1	0.871	1.999	1.128	1.71	0.289	25.6206	25.59972
SPDV1	0.873	1.867	0.994	1.61	0.257	25.8551	
SPDV1	0.872	1.954	1.082	1.68	0.274	25.3235	
SPDV2	0.871	1.116	0.245	1.053	0.063	25.7143	25.5325
SPDV2	0.887	1.064	0.177	1.018	0.046	25.9887	
SPDV2	0.882	1.119	0.237	1.06	0.059	24.8945	

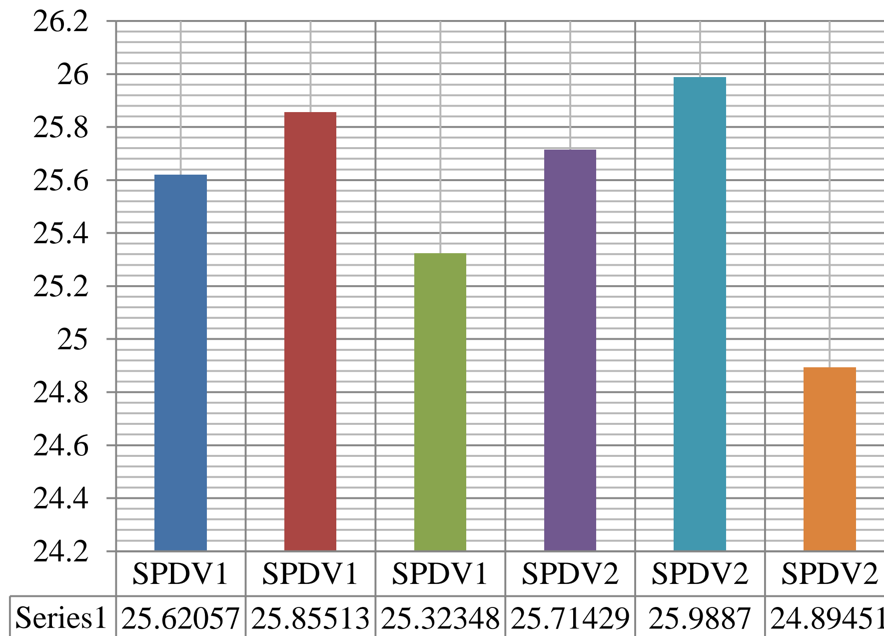


Figure 7.2: Bar diagram of the percent clot lysis by the dichloromethane fraction

The other two fractions produce weak thrombolytic activity. The average clot lysis of the ethyl acetate fraction is found to be 10.76709 for volunteer 1 and 10.66282 for volunteer 2.

Table 7.4: Clot lysis by ethyl acetate fraction of the *S. pinnata* skin

Name of Extract	**Wt of empty Eppendrof (W_1) gm**	**Wt of Eppendrof with clot (W_2) gm**	**Wt of clot (W_2-W_1) gm**	**Wt of Eppendrof after clot lysis (W_3) gm**	**Wt of released clot (W_2-W_3) gm**	**% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)**	**Average**
SPEV1	0.885	1.12	0.235	1.095	0.025	10.6383	10.76709
SPEV1	0.901	1.14	0.239	1.114	0.026	10.8787	
SPEV1	0.899	1.103	0.204	1.081	0.022	10.7843	
SPEV2	0.883	1.042	0.159	1.025	0.017	10.6918	10.66282
SPEV2	0.903	1.136	0.233	1.111	0.025	10.7296	
SPEV2	0.936	1.324	0.388	1.283	0.041	10.5670	

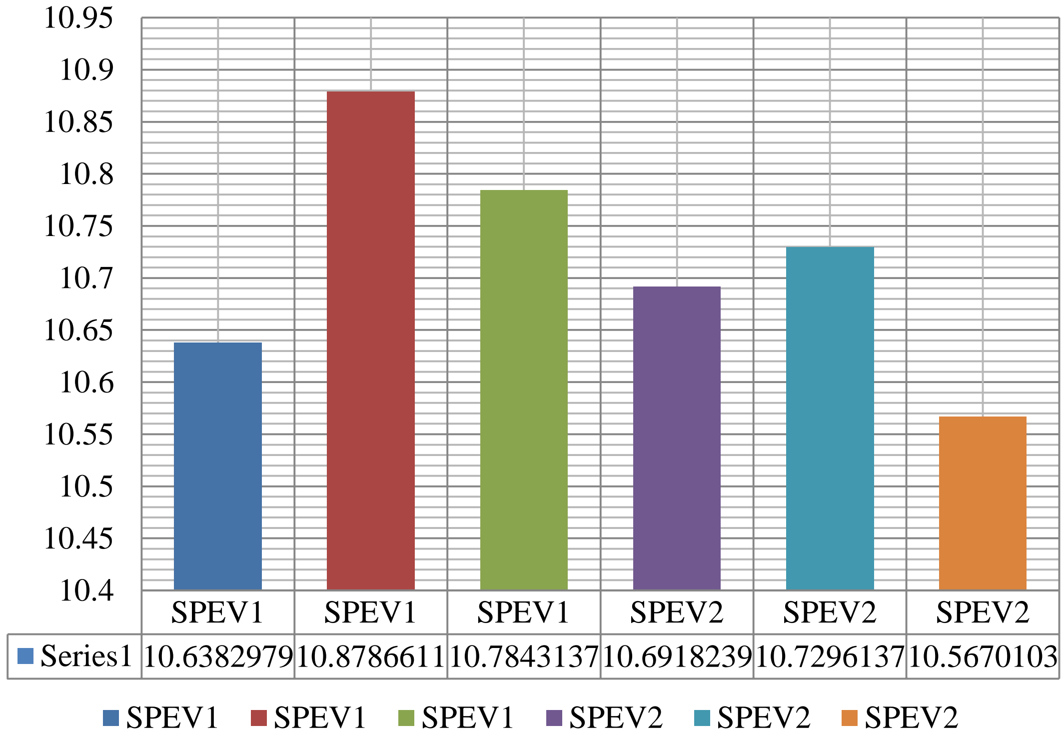


Figure 7.3: Bar diagram of the percent clot lysis by the ethyl acetate fraction

Table 7.5: Clot lysis by aqueous fraction of the *S. pinnata* skin

Name of Extract	Wt of empty Eppendrof (W_1) gm	Wt of Eppendrof with clot (W_2) gm	Wt of clot (W_2-W_1) gm	Wt of Eppendrof after clot lysis (W_3) gm	Wt of released clot (W_2-W_3) gm	% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)	Average
SPAV1	0.878	0.957	0.079	0.949	0.008	10.1266	10.2257
SPAV1	0.893	0.961	0.068	0.954	0.007	10.2941	
SPAV1	0.881	0.959	0.078	0.951	0.008	10.2564	
SPAV2	0.885	0.952	0.067	0.945	0.007	10.4478	10.21677
SPAV2	0.953	1.141	0.188	1.122	0.019	10.1064	
SPAV2	0.892	1.1	0.208	1.079	0.021	10.0962	

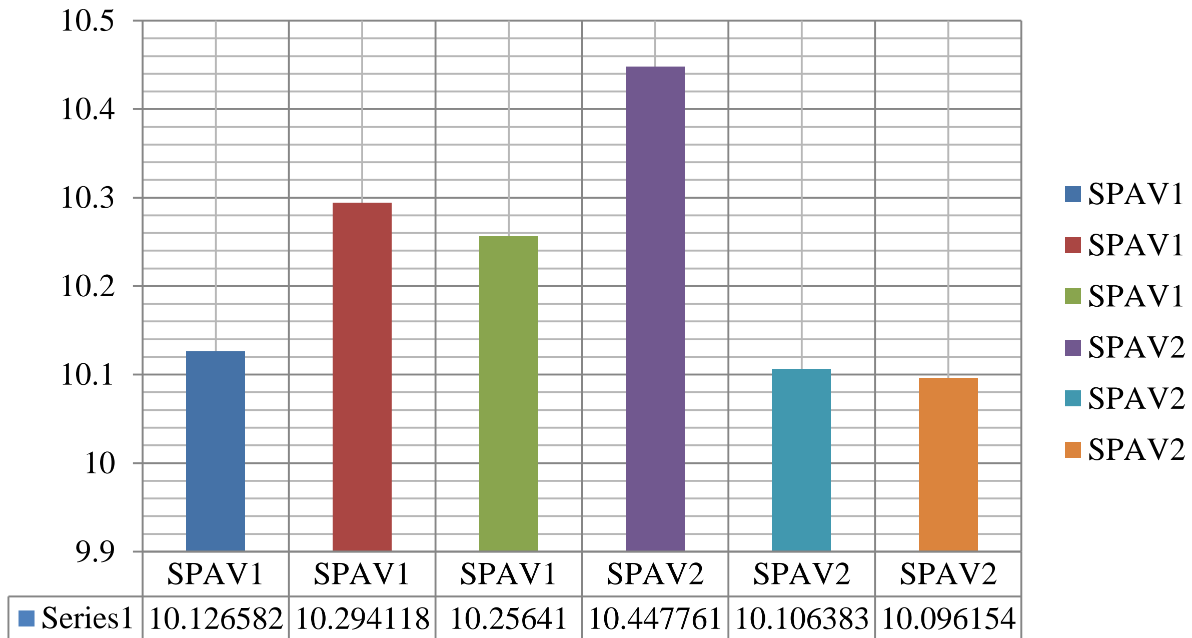


Figure 7.4: Bar diagram of the percent clot lysis by the aqueous fraction

For the aqueous fraction average of clot lysis is 10.2257 for volunteer 1and 10.21677 for volunteer 2.

The study examined the thrombolytic potential of different extracts of *S. pinnata* skin *in vitro.* It is found that among all the samples only n-hexane fraction of the ethanolic extract of the plant has thrombolytic activity compared to the standard. Again, only single concentrations of the extracts were examined. So finding of the dose related trend, if any, of thrombolytic activity can be subjected to further study.

Chapter-8: Conclusion

8. Conclusion

The *n*-hexane, carbontetrachloride, and chloroform soluble fractions of ethanoic extract of *Spondias pinnata* showed significant antioxidant, antimicrobial activity which supports the traditional use of this plant in various diseases.

The study also examined the thrombolytic potential of different extracts of *S. pinnata* skin *in vitro*. It is found that among all the samples only n-hexane fraction of the ethanolic extract of the plant has thrombolytic activity compared to the standard. Again, only single concentrations of the extracts were examined. So finding of the dose related trend, if any, of thrombolytic activity can be subjected to further study.

The plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates.

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